Transient Gene Expression Following DNA Transfer to Plant Cells: The Phenomenon; Its Causes and Some Applications.

A thesis submitted in fulfilment of the requirements for the degree of
Doctor of Philosophy in Cellular and Molecular Biology
in the
University of Canterbury.

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2000

.....ACKNOWLEDGEMENTS.....

I would like to thank Dr Jack Heinemann, Dr Colin Eady and Dr Sandra Jackson for their supervision of this project, for their criticism and their encouragement. I would also like to thank Dr Ross Bicknell for his advice and support. I acknowledge the generosity of Dr Jim Haseloff for the gift of plasmid *pBINmgfp5-ER,* Dr Ed Morgan for the gift of *Nicotiana plumbaginifolia* suspension cells and advice on their culture, Dr Steve Scofield for the gift of *pSLJ1101,* Dr Nicole Houba-Herin for the gift of *pNT103* and *pNT804*, Dr Jerzy Paszkowski for the gift of *pMDSBAR*, Dr Andrew Gleave for the gift of *pART8* and *pART7*, Dr JI Yoder for the gift of *pAL144* and Dr Bernie Carroll for information on the construction of *pSLJ3621*.

This project was made possible by funds provided by a FfRST Doctoral Fellowship provided through the New Zealand Institute for Crop and Food Research and a University of Canterbury Doctoral Scholarship.

I would like to offer my grateful thanks to all those staff and students of the Plant and Microbial Sciences Department and those staff and students at the New Zealand Institute for Crop and Food Research at Lincoln who have assisted me in various ways during the course of my research and to those who have offered their friendship, support and encouragement. In particular I would like to thank: Dougal Holmes for help with computer graphics; Ruth Butler for assistance with statistical analysis; Andrew Catenach for information and advice regarding *Hieracium aurantiacum*; Jackie Healy, Tonya Frew and Meeghan Pither-Joyce for technical advice.

Especial gratitude to my partner Bridget who held the fort during those times when I locked myself away.

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Ac	***Activator*** **transposable element**
Ds	***Dissociation*** **transposable element**
DNA	**deoxyribonucleic acid**
gfp	**green fluorescent protein gene**
GFP	**green fluorescent protein**
dpi	**days post infection**
CG	**the DNA nucleotide base sequence Cytosine-Guanine**
CNG	**the DNA nucleotide base sequence Cytosine-(Cytosine, Guanine, Adenine or Thymine)-Guanine**
RNA	**ribonucleic acid**
mRNA	**messenger ribonucleic acid**
PCR	**polymerase chain reaction**
x-glu	**5-bromo-4-chloro-3-indolyl β-D-glucuronide**
ORF	**open reading frame**
ATG	**the DNA nucleotide base sequence Adenine-Thymine-Guanine**
LB	**Luria Bertani medium**
GATC	**the DNA nucleotide base sequence Guanine-Adenine-Thymine-Cytosine**
EDTA	**ethylenediamine tetraacetic acid**
TBE	**tris(hydroxymethyl) aminomethane / boric acid / ethylene diamine tetraacetic acid**
^{32}**P-dCTP**	**radioactive cytosine nucleoside triphosphate**
ASW	**artificial seawater**
ddH_2O	**deionised, distilled water**

....ABSTRACT....

A preliminary investigation into the practicality of using *Ds* elements as vectors for onion transformation was undertaken. Transient transposase expression was used to mediate *Ds* excision following co-bombardment of a transposase expression vector and a *Ds* element into onion callus tissue. *Ds* transposition in onion was demonstrated. Further development of this transformation system was not undertaken because, at the time, the low frequency of stable transformation made further investigation impractical.

Transient transposase expression as a means to mobilise *Ds* elements for gene tagging and to study transient expression was also investigated. A T-DNA construct carrying the *Activator* (*Ac*) transposase gene was transferred to leaf discs taken from an *Hieracium aurantiacum* plant containing a chromosomal *Ds* element. Shoots were regenerated under selection for antibiotic resistance resulting from *Ds* excision. Molecular analysis suggested that regenerants carried unique transposition events. Of 84 regenerated plants, 21 (25%) did not express the T-DNA marker gene and 7 (8.3%) also lacked transposase DNA sequences. These results are consistent with the theory that expression is lost due to loss of T-DNA sequences. Potential advantages of this gene-tagging method over conventional methods are: rapid recovery of individual transposition events; regenerated plants are isogenic; gene-tagging in clonal or apomictic tissue; and the transient nature of transposase expression should facilitate the stabilisation of the transposed element.

Different factors involved in the transient nature of T-DNA expression shortly after co-cultivation were also studied by using the green fluorescent protein reporter gene *m-gfp5-ER* in *Nicotiana plumbaginifolia* suspension cell transformation experiments. It was confirmed that transiently expressed T-DNAs can be lost during growth of somatic cells. However, cell death (64% of transient expressers died) and gene silencing (21% of transient expressers retained T-DNA sequences) were more important barriers to the recovery of "stably" expressing transformants than lack of T-DNA integration (15% of transient expressers lost all T-DNA sequences). Loss of transgene expression significantly limited the efficiency of plant transformation. Understanding the causes of loss of transgene expression should lead to improved transformation strategies.

Chapter 1.

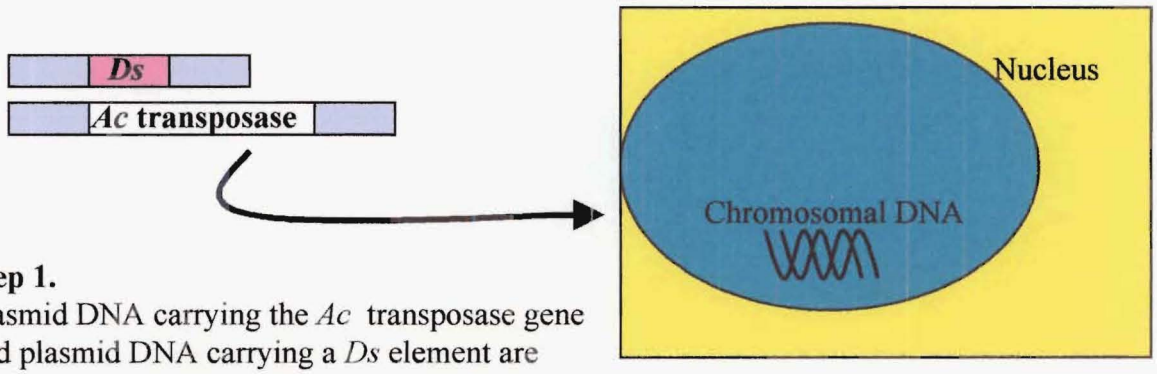
General Introduction

1. 1. Project Overview

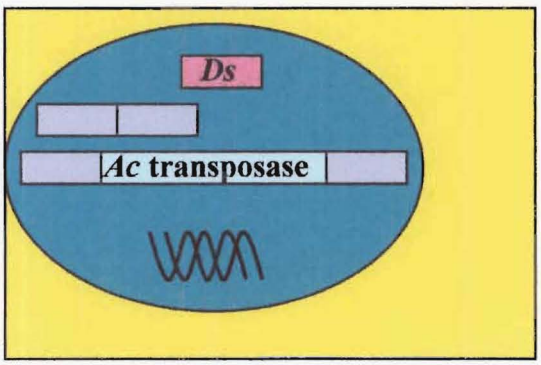
The starting point of this project was transformation of onion (*Allium cepa*). Onion is an important crop species that has been recalcitrant to transformation (40). DNA transfer to onion has resulted in a high frequency of transient expression and may result in rare stable expression events (17, 40). Possibly the frequency of stable expression might be increased by developing an efficient system for transgene integration into the onion genome. I sought to use *Ds* elements to integrate transferred DNA into onion chromosomes by transposition (Figure 1. 1. 1). Implicit in this stategy was the assumption that a lack of DNA integration was a major cause of the transient nature of transgene expression. However, while lack of transgene integration has been implicated in low frequencies of stable transformation in some systems (78), the degree to which non-integration is a barrier to stable transformation has not been determined. Hence, while an initial evaluation of *Ds* transposition in onion was carried out, a major focus of this project became determining the causes of transient expression in plant transformation. *A. cepa* was not used for these latter experiments as proven systems for culturing single cells of *A. cepa* and recovering stable transformants were not availible.

Two experimental systems were developed to investigate the causes of transient expression of transferred genes (transgenes). In the first system, transient *Ac* transposase expression in *Hieracium aurantiacum* was examined (Figure 1. 1. 2). In the second system, transient *gfp* expression in *Nicotiana plumbaginifolia* was examined (Figure 1. 1. 4). The strategy in both systems was to analyse tissue derived from cells transiently expressing *trans*-genes. The first system was also developed as a novel gene-tagging strategy (Figure 1. 1. 3). Transiently expressed *Ac* transposase was used to mobilise a *Ds* element for insertional mutagenesis, tagging the interrupted gene.

Figure 1. 1. 1.
Transposition of *Ds* elements as a strategy to transform *A. cepa.*



Step 1.
Plasmid DNA carrying the *Ac* transposase gene and plasmid DNA carrying a *Ds* element are simultaneously transferred to onion cells by particle bombardment.



Step 2. Transposase encoded by the *Ac* transposase gene catalyses transposition of the *Ds* element.

Step 3. The *Ds* element and any genes encoded within the *Ds* element are stably integrated into onion chromosomal DNA. The plasmid vectors remain extrachromosomal and are eventually degraded.

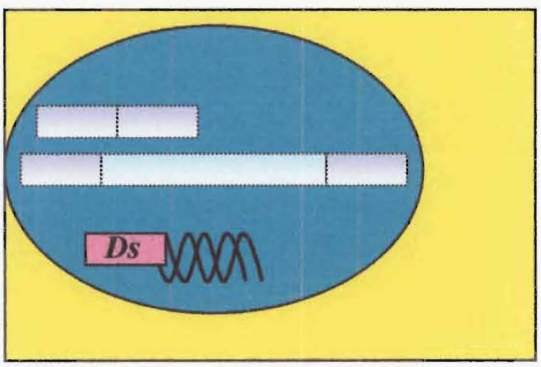
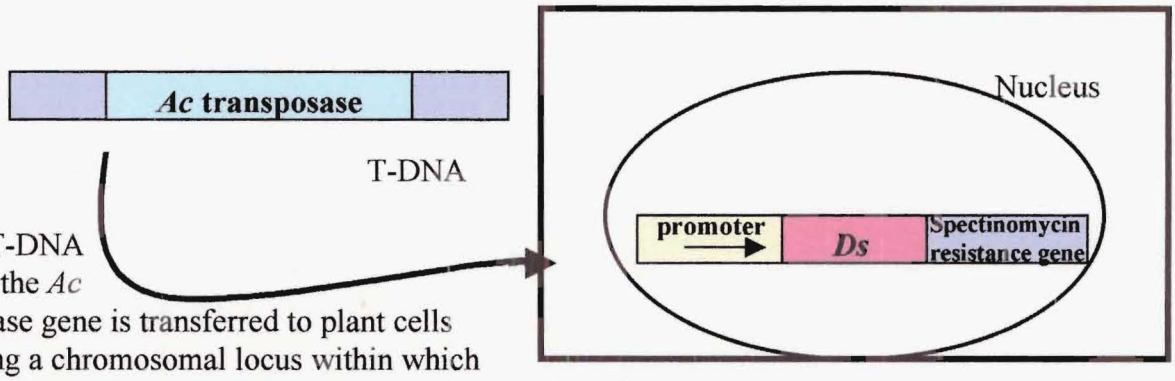
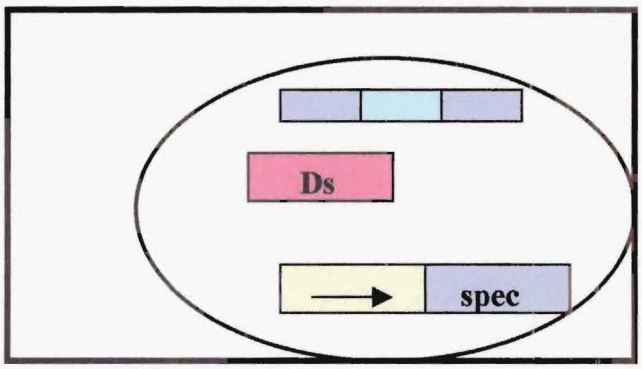


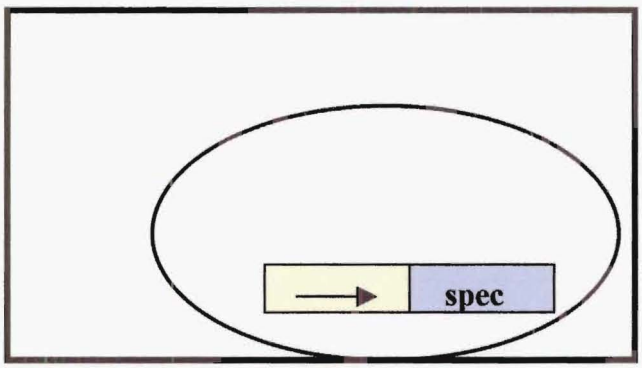
Figure 1. 1. 2.
Transposition of *Ds* elements as a strategy to recover *H. aurantiacum* cells that transiently expressed the *Ac* transposase gene.



Step 1. T-DNA carrying the *Ac* transposase gene is transferred to plant cells containing a chromosomal locus within which a Ds element blocks transcription of a spectinomycin resistance gene.

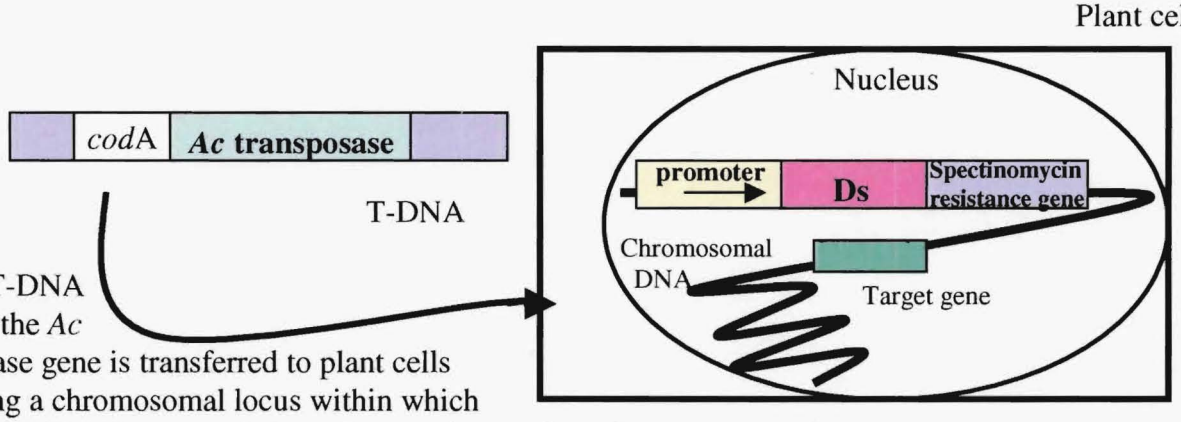


Step 2. Transient expression of the *Ac* transposase gene results in production of transposase enzyme. Transposase catalyses transposition of the *Ds* element. *Ds* excision allows expression of the spectinomycin resistance gene.

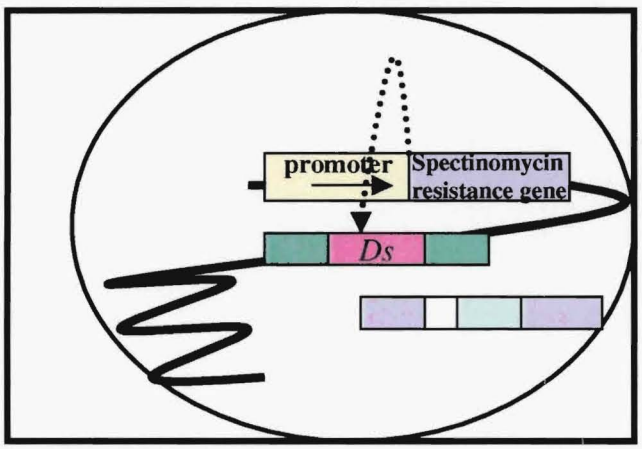


Step 3. Cells that transiently expressed the T-DNA carrying the *Ac* transposase gene can be selected on medium containing spectinomycin

Figure 1. 1. 3.
Transient *Ac* transposase expression as a method to mobilise *Ds* elements for gene-tagging in *H. aurantiacum.*



Step 1. T-DNA carrying the *Ac* transposase gene is transferred to plant cells containing a chromosomal locus within which a *Ds* element blocks transcription of a spectinomycin resistance gene. The *Ds* locus is physically linked to the target gene to be tagged.



Step 2. Transient expression of the *Ac* transposase gene results in production of transposase enzyme. Transposase catalyses transposition of the *Ds* element. *Ds* excision allows expression of the spectinomycin resistance gene. Reintegration of the *Ds* element disrupts and "tags" the target gene.

Step 3. Cells that transiently expressed the T-DNA carrying the *Ac* transposase gene can be selected on medium containing spectinomycin and will contain a stable *Ds* reintegration site. Plants that maintain the *Ac* transposase T-DNA can be eliminated due to expression of the lethal gene *cod*A also carried on the T-DNA.

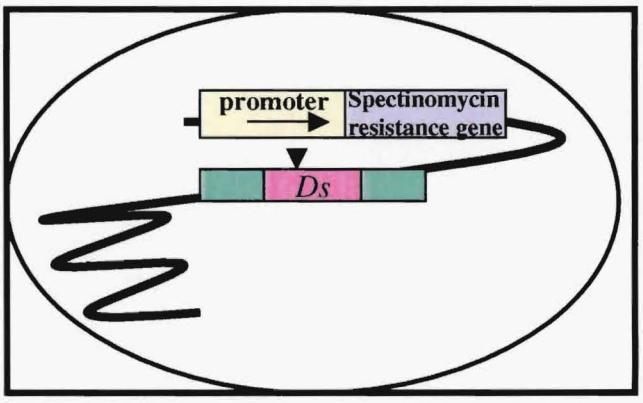
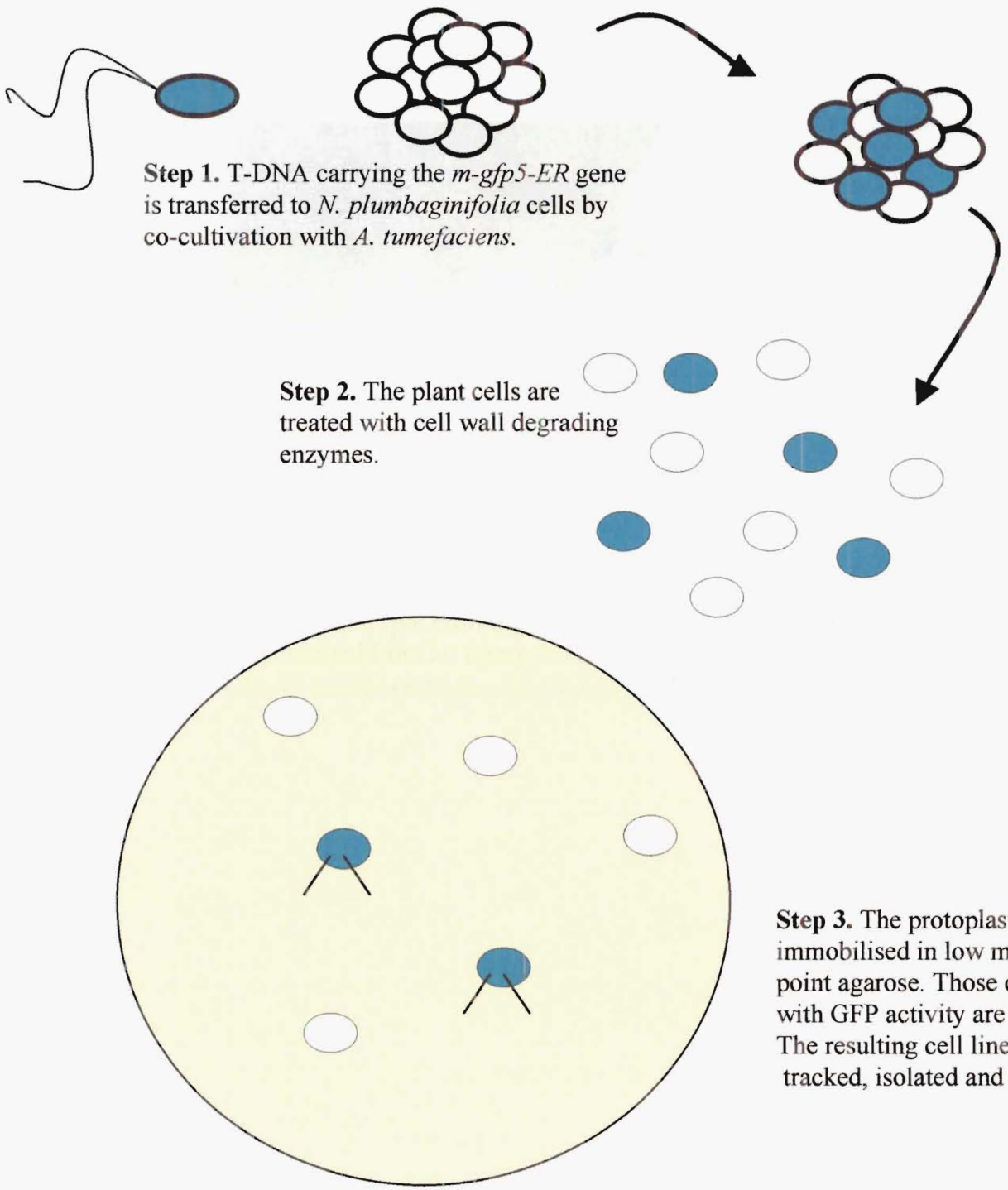


Figure 1. 1. 4.
Tracking *N. plumbaginifolia* cells that transiently expressed the *m-gfp5-ER* gene.



In the first system, the transferred DNA encoded the transposase gene from the maize *Activator* (*Ac*) transposon. The transposase gene was transferred to *Hieracium aurantiacum* cells previously transformed with a T-DNA construct that contained a *Ds* element inserted into the leader sequence of a spectinomycin resistance gene. Transient transposase expression that caused *Ds* transposition could lead to expression of the spectinomycin resistance gene. Plants were selectively regenerated from those cells that were resistant to spectinomycin (Chapter 3). Thus it was possible to enrich for cells that expressed the transferred DNA, even when that expression was only transient. However, this strategy may not have produced a random sample of transiently expressing cells if *Ds* transposition was associated with integration of the T-DNA carrying the *Ac* transposase gene.

In order to obtain a random sample of transiently expressing cells, a second system was devised. The transferred DNA contained the visible marker gene *m-gfp5-ER* (58) encoding a green fluorescent protein (GFP). *m-gfp5-ER* was transferred to *Nicotiana plumbaginifolia* suspension cells. A simple cell tracking system was devised that allowed the fate of cells that transiently expressed the *m-gfp5-ER* gene to be followed and the resulting cell lines to be isolated. Isolated cell lines were then analysed to determine whether the transient nature of *m-gfp5-ER* expression was associated with T-DNA loss or gene silencing.

1. 2. Transient T-DNA Expression in Plant Cells

1. 2. 1. The current model of transient expression

DNA transfer to plant cells typically results in a high level and a high frequency of transient expression relative to the frequency of stable expression (72, 116, 151, 168). Following transfer of double-stranded plasmid DNA to plant cells, expression of genes on the transferred DNA is presumed to be transient as a simple result of the transient presence of the extrachromosomal DNA in the plant nucleus. It is assumed that on arrival in the plant nucleus, DNA is expressed extrachromosomally. When DNA is integrated into chromosomal DNA, the cell line becomes stably transformed. DNA molecules that are not integrated are eventually lost. Although the DNA transferred from *Agrobacterium tumefaciens* (T-DNA) is thought to be single-stranded and is speculated to have a

dedicated system for integration into the plant genome, transient T-DNA expression is also assumed to be extrachromosomal. For example, Rossi et al. (124) used transient expression (measured by counting the number of cells expressing a transferred reporter gene) as an indication of the number of T-DNA molecules arriving in the plant nucleus. Referring to a T-DNA construct carrying the *npt*II gene (kanamycin resistance) and *uid*A reporter gene (visible blue colour on staining) transferred into plant tissue that was then cultured on medium supplemented with kanamycin, they state:

> The ratio between the number of calli per seedling and the number of blue spots per seedling is defined as the efficiency of integration; it reflects the proportion of the number of T-DNA molecules integrated relative to the total of the molecules entering the nucleus. (124, page 128)

Rossi et al. are bluntly stating current dogma that is generally implict and occasionally explicit in research involving transient expression.

The concept of transient expression was not always inseparable from an assumed mechanism. For example, Prols et al. (119) defined the phenomenon of transient expression:

> The phenomenon of 'transient expression' is defined as gene expression with an early maximum followed by a subsequent decline. (119, page 223)

Since the first descriptions of transient expression in plant cells, assumptions about the underlying processes have gone unchallenged. Also, there has been a subtle change in the meaning of 'transient expression'. The phenomenon originally described as transient expression was the rapid flux in gene expression that was measured as the average activity of the enzyme encoded by the transferred DNA sampled from a population of cells (eg. 161). The term "transient expression" now includes the flux in the frequency of cells that express the transferred gene (transgene) following DNA transfer (eg. 72). While the rise and fall in the frequency of expressing cells mirrors the rise and fall in average enzyme activity, it is not transient expression as defined or investigated by Prols et al. (119). It is possible, and untested, that these two phenomena are causally unrelated.

1. 2. 2. Evidence for the current model of transient expression

The idea that the transient nature of T-DNA expression resulted from elimination of unintegrated T-DNA has not been tested. The flux in average enzyme activity associated with T-DNA transfer was originally presumed not to be due to expression from integrated T-DNA because the peak enzyme activity was so high. The number of T-DNA copies integrated was thought to be too low to account for the large amount of enzyme necessary for such high activity (72). No quantitative evidence, relating the total number of integrated T-DNA copies to enzyme activity, has been presented to support this assertion. Indeed, until this work (Chapter 4), no one had isolated a random sample of transient expressers so the only estimate of the number of T-DNAs integrated into the genome had to come from the number observed in stable expressers. A low number of integrated T-DNAs in stable expressers would be predicted if transient expression was due to expression from integrated T-DNA and loss of expression was due to gene silencing associated with high T-DNA copy number. If so, transient expressers might contain sufficient silenced copies of the T-DNA to account for the high transient enzyme activity.

It was also argued that once integrated, expression of genes on the T-DNA would not vary. For example, Yoshioka et al. (168) examined transient β-glucuronidase expression after T-DNA transfer to tobacco cells. The rate of synthesis of β-glucuronidase rapidly peaked and declined from 24 to 60 hours after the start of co-cultivation. The authors argued that the kinetics of β-glucuronidase synthesis alone was evidence of the extra-chromosomal origin of transient expression as the rate of β-glucuronidase synthesis after T-DNA integration into the plant chromosomes should have been constant (168). This assertion is not consistent with the large body of literature describing gene silencing (reviewed in 96).

Elimination of non-integrated DNA is, nevertheless, a reasonable explanation of transient expression. Extrachromosomal transferred DNA is gradually eliminated from a population of transformed plant cells (eg. 145, 148, 161). The activity of an enzyme encoded on DNA transferred to plant cells has been demonstrated to peak and decline over a period of more than 10 days after DNA transfer (72, 161). This decline in enzyme activity was roughly correlated with loss of the extrachromosomal DNA from the plant cell population (161). However, if loss of transferred DNA resulted in loss of active enzyme, then transcription from the transferred DNA should have declined with a similar time course as DNA loss.

Where expression was highly transient, transcription appears to have ceased within a few hours of DNA transfer (107, 119). Blocking transcription or translation (with cordycepin and cycloheximide, respectively) in transformed tobacco protoplasts had no effect on chloramphenicol acetyltransferase (encoded on the transferred DNA) activity 1 and 4 hours after DNA transfer, respectively (119). Similarly, mRNA transcribed from T-DNA was not detected by RT-PCR for more than 1 and 3 days (respectively) after initiation of co-cultivation of *A. tumefaciens* with tobacco and maize cells (107). Also, the rate of synthesis of a protein encoded on T-DNA peaked and declined from 24 to 60 hours after the initiation of co-cultivation of *A. tumefaciens* with tobacco cells (168). It is possible that the very rapid loss of transcription could be accounted for by the loss of transferred DNA. Double-stranded plasmid DNA electroporated into maize protoplasts was degraded by 2.5 hours after transfer and undetectable by Southern analysis 1 day after transfer (148). Another group found that the quantity of plasmid DNA electroporated into maize cells declined over a few days and was not detected on day 5 (145). DNA transferred to cultured wheat cells by particle bombardment was degraded within a few hours of transfer (141). Loss of T-DNA after transfer from *A. tumefaciens* may be similarly rapid (130). However, others have suggested that electroporated DNA remained intact in protoplasts over the course of a 6 day experiment (163).

If transient expression is caused by loss of extrachromosomal DNA, then expression should be stable when the transferred DNA is capable of autonomous replication. For example, in experiments where the transferred DNA contained plant viral replication functions, the DNA should have been maintained by autonomous replication. However, in these experiments transient expression from the transferred DNA was still observed. Timmermans et al. (145) transferred plasmid vectors with or without plant replication functions to a maize cell line. In both cases the vector carried the *uid*A marker gene encoding β-glucuronidase. β-glucuronidase activity after transfer of the non-replicating vector increased from 1 to 2 days after electroporation and then gradually declined. β-glucuronidase activity after transfer of the replicating vector increased to a peak activity more than 10 fold higher than the non-replicating vector at 4 days after transfer but then declined sharply over the next few days. Inclusion of a replication function thus did not abolish the transient expression phenomenon. Although there was more stable expression (at least within the timeframe of the experiment) there was also more transient expression.

The authors attributed the decline in β-glucuronidase activity after transfer of the replicating vector to cell death. In the same study the vectors were also transferred to maize endosperm protoplasts. No peak and decline in β-glucuronidase activity was observed for either vector. The transient phenomenon was, apparently, dependent on something other than the stability of the T-DNA. Suarez-lopez and Gutierrez (140) used particle bombardment to transfer a plasmid vector carrying the *uid*A marker gene and wheat dwarf virus replication functions to cultured wheat cells. The number of extrachromosomal replicons increased rapidly to a maximum at 4 days after transfer. The number of replicons then declined slightly from 4 to 8 days after transfer. Transient expression (as measured by the number of cells with β-glucuronidase activity) increased to a maximum at 2-3 days after transfer and declined rapidly to very low levels at 5 and 8 days after transfer. The frequency of transient expression followed the same temporal profile as would be expected if the vector lacked replication functions and was rapidly degraded. These results are more compatible with loss of expression through gene silencing or cell death than with loss of expression through loss of unstable T-DNA copies.

Recent experimental work could be interpreted as supporting the idea that transient expression is caused by loss of extrachromosomal DNA. Research on an *Arabidopsis thaliana* ecotype that was recalcitrant to transformation suggested that, as is predicted from the current model of transient expression, transient expresion was not dependent on T-DNA integration (103). Nam et al. (103) examined two *A. thaliana* ecotypes in detail. Ecotypes Aa-0 and UE-1 were, respectively, susceptible and resistant to stable transformation caused by co-cultivation with *A. tumefaciens*. Tissue that was cultured without selection for 8 weeks after co-cultivation was analysed for the presence of the T-DNA by hybridisation of genomic DNA to a labelled probe. There was approximately 5 times more T-DNA detected in Aa-0 DNA than UE-1 DNA. However, there was no observed difference between the ecotypes in transient expression (measured as average enzyme activity) from the T-DNA within 7 days of co-cultivation. Similar transient expression characteristics might suggest that similar amounts of T-DNA were transferred to each ecotype. These results suggest that transient expression was not dependent on integration of the T-DNA into the host chromosomes. This result is consistent with the current model in which transient expression is the expression from unstable, extrachromosomal T-DNA. However, it is possible that transient expression was not

directly related to T-DNA transfer and it is possible that transiently expressed T-DNAs were removed after integration into the plant chromosomes.

Sonti et al. (135) described a UV-hypersensitive (*uvh1*) and a γ-hypersensitive (*rad5*) *Arabidopsis thaliana* mutant. They argued that these mutants were relatively inefficient at stably integrating T-DNA as they show reduced frequencies of stable transformation but normal frequencies of transient T-DNA expression. They simply assumed that the difference between the frequencies of transient and stable expression was T-DNA integration. Nethertheless, the fact that putative recombination deficient mutants had elevated frequencies of transient to stable expression could be interpreted as providing support for the idea that stable but not transient expression depends on integration. However, Nam et al. (104) and Preuss et al. (117) could not find evidence that *uvh1* lines were deficient for stable transformation. Nam et al. (104) suggested, on the basis of transient expression assays, that *rad5* was deficient in T-DNA uptake rather than T-DNA integration. They measured transient expression as average enzyme activity whereas Sonti et al. (135) measured transient expression as the number of expression foci per tissue area. Neither technique is a direct measure of the number of T-DNA molecules transferred, and counting the number of expressing cells may be particularly inaccurate.

Further evidence of loss of expression through loss of T-DNA comes from the recovery of clones lacking integrated transgenes from a sample of cell lines enriched for transiently expressing cells (55, 70, 150). In the *Escherichia coli* bacteriophage P1 Cre/*lox* recombination system, the product of the *cre* gene acts in *trans* to catalyse recombination between specific 34bp (*lox*) sequences. Transient expression of *cre* recombinase has been used as a method to produce site-specific integration in *A. thaliana* (150) and for removal of marker genes in tobacco (55) after *A. tumefaciens*-mediated transfer. Enrichment for cells that expressed *cre* recombinase was achieved by selection for cells containing *cre*-mediated recombination events. In this manner, stable *cre* expression was not required for enrichment for cells that expressed *cre*. Indeed, site-specific integration mediated by *cre* would create an insertion site flanked by two lox sites in direct orientation that would be unstable in the presence of the *cre* enzyme (150). Vergunst and Hooykaas (150) simultaneously transferred T-DNA carrying *lox* sites (T-DNA-*lox*) and T-DNA carrying the *cre* gene (T-DNA-*cre*) into plant cells containing a chromosomal *lox* sequence. They

recovered 15 cell lines in which the T-DNA-*lox* had integrated into the chromosomal *lox* site. 10 of the 15 cell lines did not contain T-DNA-*cre*. From this result they concluded that:

> The isolation of Cre/*lox*-mediated recombinants, lacking an integrated *cre* vector, provides direct evidence that T-strands, which are converted into double-stranded forms, and genes, located on the T-DNA, which are expressed extrachromosomally, can be lost subsequently from the cell. The transient nature of expression is thus not due to gene silencing or the absence of cell proliferation, but to loss of gene copies as assumed before. (150, page 403)

Some of these conclusions are not supported by the data presented. No experimental evidence is presented demonstrating that the T-DNA-*cre* was expressed extrachromosomally. Also, the generalised conclusion regarding "the transient nature of expression" remains an assumption. The 5 recombinants that did retain *cre* sequences were not analysed for gene silencing. Further, any cells that did not proliferate were invisible in this study as the selection system required cell proliferation. The data does demonstrate that the transient nature of expression from T-DNA can result from loss of T-DNA sequences. Gleave et al. (55), transferred T-DNA-*cre* into plant cells containing a chromosomal site flanked by *lox* sequences in direct orientation. Transient *cre* expression could catalyse lox recombination and removal of the intermediate DNA. In their experiments, 2 out of 6 recombinants lacked *cre* sequences. This result also demonstrates loss of T-DNA sequences after transient expression.

Transient expression of *Ac* transposase has been used by several different groups to mediate *Ds* integration into plant chromosomal DNA after direct DNA transfer (eg. 69, 70, 85). Houba-Herin et al. (70), simultaneously delivered a plasmid (*pAc*) carrying the *Ac* transposase gene with a plasmid (*pDs*) carrying a *Ds* element to tobacco protoplasts by polyethylene glycol-mediated DNA transfer. Of 21 cell lines in which *Ds* elements excised from *pDs* and integrated into plant chromosomes, only 2 had also integrated *pAc* sequences (70). This result demonstrates that loss of transiently expressed DNA is common after polyethylene glycol-mediated transfer. However, when El-Kharbotly et al. (41) co-cultivated potato cells containing chromosomal *Ds* elements with *A. tumefaciens* containing a T-DNA encoding *Ac* transposase (T-DNA-*Ac*), all recovered *Ds* transposition events coincided with T-DNA-*Ac* integration. Although the authors did not reveal how

many *Ds* excision events they examined (at least 4 as there were 4 experiments) their results do not suggest a high frequency of T-DNA loss after transient expression.

Examination of plants regenerated following gene transfer suggests there is a very low frequency of integrated but unexpressed transgenes (35). De Buck et al. (35) examined a system that might have resulted in enriched recovery of transient expressers. In their experiments, two T-DNA constructs carrying different selectable marker genes were simultaneously transferred into tobacco cells from separate *A. tumefaciens* strains. Stable transformation with both constructs (co-transformation) occured at a much higher frequency (21-47%) than the predicted co-transformation frequency (less than 0.01%) calculated from the transformation frequency of one construct alone (less than 1%) (35). Thus, after co-cultivation with the two *A. tumefaciens* strains, tobacco cell lines that expressed one construct had a high probability (P = approximately 0.21-0.47) of being transformed with the other construct. After co-cultivation with both *A. tumefaciens* strains, cell lines were recovered without selection and then tested for expression from the two constructs by culturing on appropriate selective media. Clones that expressed only one selectable marker were then tested for the presence of the other construct by Southern analysis. Transformants with a silenced marker gene were rare (1 transformant out of 29). The authors interpreted these results as suggesting that most integrated constructs expressed their marker genes. However, the authors do not give a frequency of transient expression with which to compare the frequency of gene silencing. Further, as our study shows (Chapter 4), transient expressors may grow more slowly than other untransformed cells and thus be under represented in such a sample. Also, the method used to enrich for transient expressers would have left unsampled those cells that simultaneously silenced both genes. Therefore, it is unlikely that De Buck et al. (35) obtained a representative sample of transient expressers.

1. 2. 3. Evidence against the current model of transient expression.

Some experimental evidence suggests that gene silencing and cell death may be primarily responsible for transient expression. That integrated constructs might frequently be silenced is suggested by the effect 5-azacytidine (permits expression from some silenced genes by demethylating DNA (112)) has on the recovery of cell lines stably expressing a

transgene after transformation. Treating tobacco leaf discs with 5-azacytidine after co-cultivation with *A. tumefaciens* containing T-DNA encoding β-glucuronidase resulted in a 4-6 fold increase in the frequency of stable β-glucuronidase activity (112). Following co-cultivation, β-glucuronidase activity was detected in 8% of cells at 3 days post infection (dpi) (112). The percentage of cells expressing β-glucuronidase was transiently high, decreasing to near 0% by 9 dpi and was 1.5% at 24 dpi. When the leaf discs were cultured on medium supplemented with 5uM 5-azacytidine, a similar transient expression phenomenon was observed but the percentage of cells expressing β-glucuronidase recovered to 10% at 24 dpi. The authors presume, but do not demonstrate, that the pertinent effect of 5-azacytidine was demethylation of the transgene and a consequent release from gene silencing. This result suggests that gene silencing was primarily responsible for the transient nature of the expression observed.

If the principle cause of transient expression is failure to integrate the T-DNA into the plant genome, then it is reasonable to expect that increased T-DNA transfer would lead to an increased chance of integration and a greater chance of stable expression. If the frequency of transient expression is a measure of T-DNA transfer (an integral assumption of the current model of transient expression), then more transient expression should be associated with more stable expression. However, higher frequencies of transient expression are not always correlated with higher frequencies of stable expression. Mozo et al. (99) examined transient and stable transformation in *Nicotiana glauca* leaf discs after co-cultivation with several *A. tumefaciens* strains. They found that an *A. tumefaciens* strain carrying a Ti plasmid with a *vir*C mutation (*vir*C mutant) caused a lower frequency of transient expression compared to the same *A. tumefaciens* strain carrying the wild type Ti plasmid. However, *N. glauca* cells co-cultivated with the *vir*C mutant did not show the typical transient response (a peak and decline in expression a few days after co-cultivation). Instead the average activity of β-glucuronidase (encoded on the T-DNA) increased linearly. The typical transient response was restored when *N. glauca* cells were simultaneously co-cultivated with the *vir*C mutant and the *A. tumefaciens* strain carrying the wild type Ti plasmid. This data is consistent with gene silencing being the primary cause of loss of expression. The more T-DNA transferred and the more cells that receive

T-DNA, the more chance there is that gene silencing is initiated. Once initiated gene silencing can then be transmitted to neighbouring cells (153).

That transient expression may not be primarily due to expression from DNA that fails to integrate and is lost is suggested by the observation that integration of transferred DNA into the plant genome can be efficient in cell lines displaying transient expression (80). When a transgene was injected into tobacco protoplasts, the transgene was expressed transiently in about 50% of surviving cells (80). When transformed cells were cultivated with untransformed cells, no stable transgene expression was observed. However, when transformed protoplasts were cultured in isolation, the frequency of stably transformed colonies was the same as the previously observed frequency of transiently expressing cells (ie. about 50%) (80). This result suggested that transgene integration was not a barrier to stable transgene expression. The transient nature of the expression was presumably a consequence of the proximity of other cells (for example, overgrowth by untransformed cells or a silencing signal transmitted by cell contact). Large quantities of DNA can be injected into cells (80) and this system might not be comparable to other procedures, such as particle bombardment or co-cultivation with *A. tumefaciens*, where only a limited amount of DNA is carried into the cell and integration may be infrequent (116). However, following particle bombardment of tobacco leaf tissue the frequency of surviving cells containing a gold particle was approximately equal to the frequency of stable transformants (71). Notably, in the tobacco transformation system, the ratio of stable to transient expression was approximately 0.8:100 (ie. 99.2% of transformed cells died). The principle barrier to stable transformation was probably not DNA integration but cell survival (71).

Three mechanisms might explain transient expression. These are gene silencing, cell death and loss of the transferred DNA. The cause of transient expression may vary depending on the DNA delivery system and cell line used. Transient expression from extra-chromosomal DNA seems more likely when the transferred DNA is double-stranded plasmid DNA than when it is single-stranded T-DNA. Some delivery systems (for example, particle bombardment and microinjection) may be more likely to cause cell death than other systems. That transient expression is expression from unintegrated DNA that is eventually eliminated is a widely held view. Indeed, the ratio of transient to stable expression has been frequently used as a measure of integration efficiency. Until this research (Chapter 4),

no one had isolated a random sample of transiently expressing cells and determined the cause of loss of expression.

1. 3. Plant Transformation

Various techniques, both vector-mediated gene transfer (using bacterial and viral plant pathogens) and direct gene transfer (using chemical, physical or electrical transfer methods), are routinely used to deliver foreign genes to plant cells (132). T-DNA transfer from *A. tumefaciens* and *A. rhizogenes* is widely used to transform susceptible (mostly dicot but increasingly monocot) plants (68, 116). Although widely used as a plant transformation system, a number of plant species cannot be routinely transformed using *Agrobacterium*-mediated transfer, and transformation competence may even vary greatly between cultivars of the same plant species (12, 103). Direct DNA transfer techniques include protoplast fusion, electroporation, chemically-induced DNA uptake, microinjection and particle bombardment (tungsten or gold particles coated with DNA are shot directly into the target tissue).

Regardless of the method of transformation, the selection of an appropriate plant tissue that is competent for transformation and regeneration, and is susceptible to an appropriate selective agent, is critical for successful production of transgenic plants. Not all cells within a tissue, or even a seemingly homogeneous cell culture, will be competent for transformation (9, 35). However, competence for transformation may be, to some extent, a manipulable metabolic function of the cell that is determined by the cell cycle (9, 98, 152). Ideally, a tissue that contains many cells that are competent for both incorporation of foreign DNA and regeneration should be used for plant transformation. Some tissues that are highly competent for both regeneration and cell transformation may still be recalcitrant for the recovery of transformed plants due to the cells that are competent for regeneration not being competent for transformation and vice versa (53). For example, following co-cultivation of *Kohleria* internodes with *A. tumefaciens*, T-DNA expression was primarily observed in vascular tissue while shoot regeneration specifically occured from the basal cell of glandular trichomes (53). Besides regeneration and transformation competence, other aspects of tissue culture need to be considered. For example, there is a high occurance of soma clonal variation amongst plants regenerated from cultured cells (eg.

calli, suspension culture and protoplast culture) (12). Also, plants regenerated from transformed organised tissue may be composed of a mixture of transformed and untransformed cells (13, 154). A correlation between the developmental age of the tissue and transformation efficiency has been observed (eg. 52). This correlation could mean that rapidly dividing, undifferentiated, developmentally immature embryogenic cells are generally more suitable for successful transformation than other tissue types (13, 36, Eady pers. comm.).

The susceptibility of plant tissue to transformation can be improved. Pre-culturing plant tissue on regeneration medium for a few days prior to gene transfer has been used to increase the transformation frequency (52, 91, 133). Pre-culturing may increase stable transformation by increasing the proportion of regenerable cells in the tissue (91). However, pre-culturing also increases the number of cells transiently expressing the transgene (52, 133) suggesting that DNA transfer or expression is also enhanced. As there is a relationship between plant transformation and cell cycle stage (36, 152) it is possible that the pertinent effect of pre-culturing is causing cells to restart the cell cycle. No transient expression was observed in plant cells when the cell cycle was blocked prior to the S-phase (DNA replication) by culturing cells on medium containing mimosine (152). Blocking the cell cycle after DNA replication by culturing cells on medium containing colchicine only slightly reduced the frequency of transient expression (152). These results suggest that between the start of S-phase and cell division is a critical time for cell transformation.

Plasmolysis of cells in an osmoticum has been shown to dramatically increase the frequency of transient expression (91, 105, 149, 164). It is assumed, but not demonstrated, that the pertinent effect of cell plasmolysis is that it prevents the loss of protoplasm from cells damaged during transformation (149). However, in one study, osmotica were reported to decrease the frequency of stable transgene expression where transient expression was enhanced (106). If plasmolysis merely protected cells from physical damage it would not improve transient expression while decreasing stable expression. Osmoticums are principally, but not exclusively, used in transformation by particle bombardment (91, 106, 149).

1. 4. T-DNA Transfer from *Agrobacterium tumefaciens*

A. tumefaciens is a plant pathogen that transfers DNA to plant cells causing tumours on dicotyledonous plants (81). DNA transfer from bacteria to plants may be functionally analogous to DNA transfer between bacteria (7, 63, 138, 139). The transferred DNA (T-DNA) is defined by 25bp direct repeats (left and right border sequences) and is located on a large, extrachromosomal plasmid called Ti. The Ti plasmid also contains the virulence (*vir*) region that encodes genes involved in T-DNA transfer (136). The natural process of T-DNA transfer has been modified for use as a gene delivery technique in biotechnology and gene research. This has been achieved by creating a binary system where the gene of interest is inserted between right and left border sequences on a small plasmid separate from, but co-resident with, the Ti plasmid in *A. tumefaciens*. The Ti plasmid carries the *trans*-acting functions necessary for T-DNA transfer but usually does not contain functional T-DNA sequences.

The *vir* region has 6 complementation groups (*vir*A, *vir*B, *vir*C, *vir*D, *vir*E, and *vir*G) (135). Additional *vir* loci such as *vir*F are specific to various types of Ti plasmids and appear to act to determine the host-range of *Agrobacterium* genetic colonisation (67, 73, 121). *vir*A and *vir*G are constitutively expressed genes encoding proteins that form a two component regulatory system that detects compounds associated with damaged plant cells (87, 113, 136). Along with additional regulators encoded on the chromosome, *vir*A and *vir*G act to induce expression of the other *vir* genes (30, 87, 113).

After *vir* gene induction, three T-DNA intermediates are detectable in the *A. tumefaciens* cell. These are single-stranded linear T-DNA, double-stranded T-DNA circles, and double stranded T-DNA linear molecules (29, 81). However, only single-stranded T-DNA molecules have been detected in the plant cell after transfer (170). An experiment where transferred DNA strands had to recombine to restore marker gene expression, also suggested that single-stranded T-DNA molecules were transferred to plant cells (146). As inversion of the right border leads to reduced virulence and transfer of most of the Ti plasmid, it is thought that the single-stranded T-DNA molecule is formed by strand displacement from right to left from a single-strand nick at the right T-DNA border to a single-strand nick at the left border (158, 171). This polarity of transfer is thought to be due to the sequence context of the borders (155). VirD1 and VirD2 are required for the

formation of single-strand border nicks (48, 137) and, at least within the bacterial cell, a VirD2 molecule is bound to the 5' end of the displaced strand (66, 159, 169). Although protein transfer during conjugation between bacteria has been demonstrated (62) there is no direct evidence that *vir* proteins are transferred during T-DNA transfer from bacteria to plant cells.

1. 5. Integration of T-DNA into the Plant Genome

Foreign DNA is usually integrated into plant nuclear DNA by illegitimate recombination with very short stretches of DNA sequence similarity (54, 95, 97). However, transgene integration into the nuclear genome by homologous recombination may also occur at a low frequency (86, 122). Foreign DNA introduced to the plant plastid genome has been observed to integrate by homologous recombination (20, 56). The mode of T-DNA integration is also probably dictated by host factors as T-DNA is preferentially integrated by homologous recombination in yeast and by illegitimate recombination in plants (16, 54, 95, 97). As low level irradiation and treatment with ultra-low doses of bleomycin induces both DNA repair and foreign DNA integration in plant cells, the two processes are possibly related (9, 34, 78, 151). Efforts to characterise plant mutants that are deficient for foreign DNA integration should help elucidate the pathways of DNA integration (102, 103, 104).

The mechanisms of T-DNA nuclear uptake and integration are the subject of considerable current research (reviewed in: 151, 173). The T-DNA is probably primarily transferred to the plant cell as single stranded DNA (only single stranded T-DNA molecules have been detected in plant cells co-cultivated with *A. tumefaciens* (146, 170)) covalently bound at the 5' end to VirD2 protein (VirD2 is bound to the 5' end of the T-DNA in *A.tumefaciens* and *E. coli* (66, 159, 170)). Based on the *in vitro* ssDNA-binding properties of VirE2, it is thought that VirE2 proteins may also bind to the T-DNA either in the plant cell or prior to transfer (26). Indirect evidence suggests that putative nuclear localisation sequences (NLS) on VirE2 and VirD2 help transport the single-stranded T-DNA to the plant nucleus after co-cultivation (27, 28, 124, 125, 172). VirE2 and VirD2 proteins expressed in plant cells as translational fusions to β-glucuronidase accumulate in the plant cell nucleus (27, 28). Further, protein fusions between β-glucuronidase and the putative VirE2 NLSs expressed in tobacco cells are transported to the plant nucleus (28). VirE2 protein expressed in

tobacco cells complemented a *vir*E mutation in *A. tumefaciens*, completely restoring tumourigenicity (28). Assuming that VirE2 was not transported to the *vir*E mutant bacterial cell during co-cultivation, this result suggests that the function of VirE2 can be carried out in the plant cell and is consistent with transfer of VirE2 to the plant cell during T-DNA transfer. However, given that VirE2 expressed in plant cells was only detected in the nucleus (28) it seems surprising that VirE2 expressed in plant cells can completely complement a bacterial *vir*E mutant if the relevant role of VirE2 is T-DNA transport through the cytoplasm.

While it is generally accepted that the transferred DNA is primarily single stranded, it is uncertain whether the T-DNA is single stranded or double stranded immediately prior to integration (54, 143, 170). While single stranded T-DNA is detected in plant cells immediately after co-cultivation, transient expression (that is presumed to be extrachromosomal) must, given the orientation of some transgenes, originate from transcription of double stranded T-DNA (72, 107, 168, 170).

T-DNA integration into nuclear DNA is random, possibly with some preference for integration into actively transcribed DNA (1, 24, 25, 65, 80). It has been argued that T-DNA integration may, in part, be mediated by VirD2 (101, 107, 147). Specific mutations to the omega region of the VirD2 protein reduce stable T-DNA integration, possibly without a corresponding reduction in the efficiency of right border cleavage or nuclear localisation of the T-DNA (101, 107). Narasimhulu et al. (107) have shown that a specific mutation in the *vir*D2 omega sequence decreases the ratio of stable expression to transient expression in tobacco suspension culture cells when compared to expression following co-cultivation with an *A. tumefaciens* strain containing an unmutated *vir*D2 gene (107). On the (unproven) assumption that the difference between the frequency of transient expression and stable expression is the frequency of T-DNA integration, Narasimhulu et al. (107) argued a role for VirD2 in T-DNA integration.

This work was extended in a later paper where Mysore et al. (101) demonstrated that they could not detect transient expression from a promoterless *uid*A gene in tobacco cell cultures after transfer (detected by RT-PCR amplification of a mRNA encoded separately on the T-DNA) from a *vir*D2 omega mutant *A. tumefaciens* strain. They argued that this

result suggested VirD2 was involved in integration as the promoterless *uid*A gene required integration for expression. However, no evidence was presented to show that there was actually a difference in the number of promoterless *uid*A expression events observed after transfer from the *vir*D2 omega mutant and the number that would be expected if VirD2 was not involved in integration. They also show that 42 days after cocultivation of tobacco suspension cells with the *vir*D2 omega mutant *Agrobacterium* strain, no T-DNA was detected in tobacco genomic DNA (101). However, this result is hard to interpret without a comparison with a co-cultivation system with similar transfer frequencies but wild-type integration. A role for VirD2, or any other bacterial protein in T-DNA integration, remains to be demonstrated.

1. 6. Epigenetic Modification of Transgene Expression

Integration of transgenes into the host genome does not ensure stable expression. Within the host plant cell, transgene expression can be reduced or stopped without changes to the DNA sequence, a phenomenon called gene silencing (140). Gene silencing can be broadly categorised as being either transcriptional gene silencing or post transcriptional gene silencing. In practical terms, these categories are usually defined by the results of nuclear run on assays that determine whether or not efficient transcription is possible from the silenced transgene *in vitro*. Both transcriptional gene silencing and post transcriptional gene silencing have been associated with cytosine methylation (8, 33, 43, 114). Promoter and transgene CG and CNG sequences are the major substrates for cytosine methylation in plants but other sequences are also targets for methylation (93, 114, 123). DNA hypermethylation of promoter and gene sequences is associated with alterations in chromatin structure that obstruct transcription (76, 167).

The plant genome consists of regions distinguishable by structure, sequence and transcriptional activity (19, 118). Differences in expression from homologous T-DNAs integrated at different sites in the plant genome suggested that gene silencing might sometimes simply result from the chromosomal position of the integrated DNA (5, 118). In other instances, gene silencing may reflect more active processes. Some epigenetic phenomena, such as the sequence-specific degradation of mRNA, may protect the host from invasive viral RNA (120, 126). Although untested, it has been suggested that high

gene expression, structural features (eg, integration intermediates, alteration to chromatin structure), sequence features of the transgene, and disruptions to the normal genome function might trigger gene silencing (42, 82). The transgene or its promoter may pair with homologous DNA or RNA sequences creating structures that are methylated (43). Such structures may be common where multiple transgenes integrate into the same locus (96, 127, 167). Multi-transgene loci have been shown to be associated with changes in the local chromatin structure (more condensed chromatin as judged by DNaseI digestion), DNA methylation and gene silencing in *Arabidopsis* (167).

A silenced transgene can impose the silent phenotype on a homologous gene on a separate DNA molecule (8, 32, 96). Homologous transgene loci or an endogenous locus and a homologous transgene locus can also interact to produce co-ordinate inactivation of both loci (31, 75). *Trans*-inactivation requires sequence similarity and may depend upon DNA-DNA, RNA-RNA or DNA-RNA interactions (32, 144). Genes transferred into plants containing the 271 silencing locus (repeated copies of a plasmid carrying the *nii*I transgene driven by the 35S promoter) are silenced if they have sequence similarity with either the 35S promoter or *nii*I gene (144). Silencing appears to depend upon nucleic acid pairing as sequence similarity sufficient to allow pairing is required for silencing. A promoter with sequence and structural similarity to the 35S promoter, predicted to bind the same *trans*-acting factors but with insufficient similarity to allow DNA-DNA pairing with the 271 locus, was not silenced when transferred to a plant containing the 271 silencing locus (144)

Post transcriptional gene silencing initiated at a local site within the plant body can be spread from cell to cell to become systemic (110, 111, 153). The maintenance of silencing depends on the presence of the tissue that initiated the silencing. This dependence is demonstrated by grafting experiments where silenced tissue grafted on to unsilenced root stock leads to systemic silencing. Removal of the graft can then result in gene expression in the root stock (111). It has been speculated, from the grafting experiments, that there is a propagatable, sequence-specific signal for RNA degradation that is responsible for systemic silencing (111, 160).

Some aspects of gene silencing make it a likely mechanism for turning off transient expression shortly after gene transfer. As gene silencing may be initiated by DNA-DNA or

DNA-RNA interactions between homologous sequences, the occurrance of numerous, homologous, extrachromosomal DNA molecules after DNA transfer possibly provides an opportunity for the establishment of gene silencing. Also, if gene silencing is, as suggested, partly a viral protection system, and given possible similarities between transferred DNA and viral DNA (highly expressed, extrachromosomal genetic elements) DNA transfer might be expected to trigger a silencing response.

In order to account for the transient nature of expression, gene silencing would have to be initiated rapidly (within hours) after DNA transfer. T-DNA carrying *gfp* sequences with or without an upstream promoter triggered the silencing of a chromosomal *gfp* gene rapidly after DNA transfer (153). When a single leaf on a tobacco plant (containing a chromosomal *gfp* gene) was infiltrated with *A. tumefaciens* (containing T-DNA carrying a *gfp* gene), silencing of the chromosomal *gfp* gene was initiated within the leaf and spread gradually throughout the plant (153). When the infiltrated leaf was removed 2 days after the start of infiltration, silencing still spread throughout the plant body in some instances. This result demonstrated that a silencing signal was translocated out of the leaf within 2 days of the start of infiltration, suggesting that gene silencing could be initiated within that period.

1. 7. Improved Integration Strategies

Attempts have been made to improve the frequency of transgene integration and stability of transgene expression following direct DNA delivery (60, 69, 70, 85). "Agrolistic" transformation involves biolistic delivery of a gene flanked by Ti-plasmid right and left borders (60). This construct is co-delivered with the Ti-plasmid *vir*D2 and *vir*D1 genes. The assumption behind the agrolistic strategy is that, following transient expression of the *vir* genes, the VirD1 and VirD2 proteins will act at the left and right borders *in planta* and catalyse the excision of the T-DNA from the plasmid vector and its integration into the plant genome. The anticipated result is a hybrid system free from the host range limitations of *Agrobacterium*-mediated delivery while retaining *Agrobacterium*-mediated integration (60).

The agrolistic system has been used to transform tobacco cells (60). Hansen and Chilton (60) claimed that agrolistic transformation resulted in a lower transgene copy number per transformation event than non-agrolistic particle bombardment. However, their initial experiments found that more than 90% of the transformed calli contained integration events that probably were not mediated by the VirD1 and VirD2 proteins. The validity of Hanson and Chilton's claims for their system is clouded by a lack of published data. Narayan et al. (108) explored the possibility of using an agrolistic system to transform *Pinus radiata* cells. Rather than expressing *vir*D2 and *vir*D1 genes *in planta*, they planned to coat T-DNA with VirD and VirE proteins, *in vitro*, prior to biolistic delivery to plant cells (108). No further published data is currently availible on this technology.

Another approach to improving the frequency and precision of foreign gene integration utilises plant transposons. *In planta* transposition of a foreign gene from a plasmid to the plant genome might produce high frequency integration of the desired sequence at independent, low copy number, loci. Initial work on transposon-mediated transgene integration in plants focused on the maize transposable element *Ac* and its nonautonomous derivative *Ds* (69, 70, 85). One peculiarity of *Ac/Ds* transposition may, however, detract from it as a system to transfer transgenes from transformation vectors to plant chromosomes. *Ac/Ds* elements predominantly transpose short distances, possibly with a requirement for physical contact between donor and recipient sites (45, 83). A more effective transfer vehicle might be provided by a transposon system, such as the *Mutator* transposable element, which displays a preference for long distance transposition (90).

In 1990, Laufs et al. (84) suggested that *Ac/Ds* transposons could be used as vectors for plant genetic engineering. They inserted non-autonomous *Ds* transposons into the genome of the wheat dwarf virus and introduced the resulting vector into *Zea mays* protoplasts. *Trans*-activation with *Ac* transposase resulted in *Ds* excision from the viral vector. Their results suggested that, as for chromosomal *Ac* elements, excision was linked to replication of the *Ds* locus (ie. viral replication). However, later work by Houba-Herin et al. (70) suggested that, following vector delivery, *in planta* replication of the vector was not essential for *Ac* transposition. The precise relationship between transposition frequency and vector replication has not yet been ascertained (85, 162).

Houba-Herin's research group co-delivered a *Ds* plasmid vector and an *Ac*-transposase expression vector into *Nicotiana plumbaginifolia* protoplasts by polyethylene glycol-mediated DNA transfer. *Trans*-activation by *Ac* transposase resulted in excision of the *Ds* element from the plasmid and *Ds* integration into the plant genome (70). Of 32 plants regenerated from transformed tissue, 24 showed only transposon-mediated integration events (11 had a single chromosomal *Ds* element, 2 had two chromosomal copies and 11 had three or more copies). The 8 remaining transgenic plants contained multiple plasmid insertions that did not result from transposition events. *Ds* excision from a plasmid vector appeared to result in the precise integration of the entire *Ds* element at random positions in the chromosomes of *N. plumbaginifolia* protoplast-derived cells (69). Similar results have been obtained in *N. tabacum* and *A. thaliana* protoplasts (85). In *N. tabacum* and *A. thaliana, Ds* elements were capable of transferring long stretches (~10kb) of foreign DNA from plasmid vectors to the plant chromosomes (85).

Finally, site-specific recombination has been explored as an integration strategy that would allow the precise integration of a defined sequence of foreign DNA into a predetermined plant chromosomal locus. Site-specific integration can be achieved using the plant's homologous recombination system (20, 56). While homologous recombination within the plant nucleus may be rare (86, 122), homologous recombinatioin can be used to target DNA integration to specific sites within the plant plastid genome (20, 56). The *E. coli* bacteriophage P1 *cre/lox* recombination system has been used to integrate DNA at specific sites on plant chromosomes (150). In the *cre/lox* system, the product of the *cre* gene acts in *trans* to catalyse recombination between specific 34bp (*lox*) sequences. Where *lox* sites are carried on both the transferred DNA and a plant chromosome, *cre* recombinase can catalyse site-specific DNA integration.

1. 8. *Activator/Dissociation* Transposons

Ac belongs to a "family" of eukaryotic transposable elements that includes transposons from animals, such as *hobo* (fruit fly), and transposons from plants, such as *Tam3* (snapdragon) (44). These elements share several characteristics such as short terminal inverted repeats with sequence similarity and a similar mode of transposition eg. non-replicative transposition and the creation of 8bp target site duplications. The similarity in

sequence of the transposase genes from some of these elements has been put forward by some researchers as evidence that these mobile elements are homologous (3, 18, 44). Aside from the autonomous *Ac* element, there are three classes of non-autonomous (*Ds*) elements that can be mobilised in *trans* by *Ac* transposase: 1) *simple Ds* elements that are deletion derivatives of *Ac*; 2) composite *Ds* elements that contain rearranged *Ac* sequences with some sequences unrelated to *Ac*; 3) *Ds1* elements that have only two short (13bp and 26bp) terminal sequences with similarity to *Ac* (83). Gene integration exploiting *Ac/Ds* elements has, to date, focused on simple *Ds* elements. A promoter-*Ds1-uid*A construct has previously been used to examine *Ds1* excision (46). *Ds1* elements and simple *Ds* elements may behave differently as integration vectors.

The *Ac* element is 4.5kb long, encodes a single protein (the *trans*-acting transposase) and has 11bp imperfect terminal repeats (reviewed in 47). Approximately 15 AAACGG motifs and similar sequences are scattered within 200bp of each inverted repeat. The *Ac* transposase binds weakly to the inverted repeats and with high affinity to the AAACGG motifs *in vitro* (6). *Ac* transposes by a non-replicative, cut and paste, method (129). As well as *trans*-activating transposition, the transposase protein is apparently capable of negatively regulating transcription of the transposase gene in at least some plant species (51). Furthermore, the activity of *Ac* transposase may not be directly dose dependent (63). In maize, a transposase concentration threshold exists above which transposase activity is inhibited (63) possibly by transposase aggregation (45). However, Swinburne et al. (143) found no evidence that high levels of transposase inhibited *Ds* transposition in *A. thaliana*. Scofield et al. (128) suggested that inhibitory concentration thresholds are an intrinsic property of *Ac* transposase but that those thresholds vary between plant species.

1. 9. *Dissociation* as a Vector for *Allium cepa* Transformation

There is currently no published data on *Ac* transposition in *A. cepa*. Since *Ac* is able to transpose efficiently in a wide range of unrelated plants, host proteins essential for transposition must be widespread in the plant kingdom (22). Host proteins are thought to be involved in the repair of the donor site following *Ac* excision (129). Host proteins may also be involved in the formation of the transposition complex (22). A sequence motif (GGTAAA) that is repeated in both termini has been found by gel mobility shift assays to

provide binding sites for, as yet, uncharacterised plant nuclear proteins (89). The GGTAAA motif is also present in the terminal regions of other plant transposons, perhaps suggesting a function in transposition (89). As *Ac* transposition occurs during or shortly after host DNA replication (23, 57), host proteins are likely to play a part in the regulation of transposition. This regulation may involve different binding patterns of *Ac* transposase depending on methylation patterns of *Ac* (156, 157).

Where *Ac/Ds* elements are used as transformation vectors it is important that the target plant does not contain an endogenous transposase capable of mobilising the element as stable transgene insertion is desirable. Transposons homologous to *Ac* are widespread among the eukaryota (44). The *Alliums* are not devoid of transposons (15) and may contain elements encoding *trans*-acting factors capable of mobilising *Ds* elements or other non-autonomous transposons. Cross-mobilisation, by endogenous factors, of transposons introduced to unnatural hosts is not unprecedented. For example, the narrow-host-range *Drosophila melanogaster hobo* transposable element can be mobilised by endogenous transposases in non-host *Drosophila* species (59), in *Musca domestica* (3) and in a large selection of *Tephritid* insects (58). While *Ds* elements that have been used as integration vectors have proven to be stable once integrated (132), potential *Ds* instability should be considered before using non-autonomous transposable elements as integration vectors in *Alliums*.

1. 10. *Dissociation* Elements and Gene Tagging

Gene tagging is used to identify DNA sequences responsible for a specific phenotype. Random mutagenesis by insertion of DNA elements into the genome can simultaneously modify the plant phenotype and mark the mutated gene responsible for that phenotype. The *Ac/Ds* transposable element system from maize has been used in attempts to isolate genes by insertional mutagenesis in a wide variety of plants, for example: rice (132, 142); tomato (21); lettuce (166); *Arabidopsis* (4); and flax (49). Gene tagging strategies based on *Ac/Ds* typically exploit the dependence of *Ds* mobility on the presence of the *trans*-acting factor to provide some control over transposition. The *Ds* element is used as the transposable element while the *Ac* transposase gene is encoded on a separate construct. The elements of this binary system are transferred into separate cells and transformed plants regenerated.

Crossing the two transformed plant lines brings the elements together. The resulting seedlings are selected for *Ds* excision and screened for phenotypes suggesting insertional mutagenesis of the target locus. Subsequently, the *Ds* insertion can be restabilised by segregating the two unlinked elements. Separating the tissue culture steps involved in transformation and regeneration from selection for *Ds* transposition, helps avoid confusing mutations caused by *Ds* insertion with somaclonal variation (4).

Transferring both elements of the binary system into the same cell (co-transformation) has been mooted as a more efficient gene-tagging strategy than crossing regenerants from two separate transformation events (50, 64). One perceived advantage of co-transformation is that it eliminates one generation, thus saving time (50). Co-transformation may also be useful in asexual systems and to facilitate gene tagging in an isogenic background where breeding incompatibility requires crossing to a genetically distinguishable strain. It has also been suggested that in most cases of co-transformation, the *Ds* element is mobilised by transient transposase expression and the transposase construct does not integrate (64, 142). Consequently, mutations caused by *Ds* insertion will be stable, facilitating genetic characterisation (64). However, co-transformation has previously resulted in a high frequency of tandem co-integration of constructs at the same locus in some delivery systems (eg. 33). Tandem co-integration could make it difficult to segregate the transposed *Ds* element away from the transposase construct. Also, transformation itself is capable of creating mutations through integration and through repair of integration intermediates (94). Such lesions and mutations unlinked to the *Ds* integration site could complicate any attempt at gene tagging. Further, a particular advantage of the *Ac/Ds* system is that the transposable element preferably transposes to sites linked to the excision site in some plant species (74, 77). This property of *Ac/Ds* has been successfully exploited by genetically linking the *Ds* excision locus to the target site, thus maximising the probability of tagging the gene of interest once the transposon is mobilised. Transferring both elements simultaneously into the same cell does not allow selection of plants with a stable *Ds* excision locus linked to the target gene prior to mobilising the *Ds* element.

In this thesis a new gene tagging strategy is described. By using transient transposase expression to mobilise a chromosomally-inserted *Ds* element it may be possible to exploit some of the advantages of a co-transformation gene tagging system (stabilisation of the

reintegrated *Ds* element through loss of transposase expression, gene tagging in an isogenic background, rapid generation of independent transposition events without the requirement of a sexually mature plant) while still being able to genetically link the *Ds* excision locus to the target gene prior to gene tagging. This strategy does, however, retain some of the problems (mutations caused by transformation and somaclonal variation) associated with gene tagging through co-transformation.

1. 11. Transformation and Tissue Culture of *Allium cepa*

Onion (*Allium cepa* L.) is an important crop species. It is subjected to intensive research and development of methods for *in vitro* regeneration, propagation and transformation (37, 38, 39, 40). Procedures for reliably transforming *A. cepa* have recently been developed (40). Using co-cultivation with *A. tumefaciens,* T-DNA constructs containing the *m-gfp5-ER* gene have been successfully delivered to immature embryo cells and whole plants regenerated under selection for resistance to the antibiotic geneticin (40). Particle bombardment has also previously been used to successfully deliver DNA constructs to *A. cepa* tissue (39). Particle bombardment and co-cultivation with *A. tumefaciens* resulted in a high frequency of transient transgene expression but a low frequency of stable expression. Transient expression assays have been used to optimise delivery conditions, to test the efficiency of various promoters and to explore the effect of tissue age and type on gene delivery and expression (39). Immature embryos were found to be more competent than microbulbs at accepting and expressing a transgene construct. Of the promoters tested, the 35S cauliflower mosaic virus promoter drove the highest level of expression.

Previous work has identified the antibiotics hygromycin and geneticin and the herbicide phosphinothricin as being effective selective agents in onion (38). Eady's review (37) of *A. cepa* transformation identified embryo/seedling-derived callus and basal plate-derived callus as being the most efficiently regenerating callus system. Other culture material identified as being potentially useful in transformation studies included microbulbs (dedifferentiated, actively dividing cell masses induced in seedlings germinated on medium containing picloram), twin scales and split stems. Current work in our lab has focused on transformation of, and regeneration from, immature embyos, and transient expression studies in cultures derived from immature embryos. Despite optimisation of

tissue culture conditions, transformation and selection, the transformation frequency is very low (maximum frequency of 2.7 stable transformants per 100 embryos). However, the transient expression frequency is several orders of magnitude higher than the stable transformation frequency. Therefore, it is clear that the mechanisms by which expression is lost are important barriers to stable transformation in onion. To address the problem of loss of expression, we have explored the feasibility of using *Ds* transposition to improve the frequency of integration of transferred DNA. We have also investigated transient expression in a model plant to try to gain an understanding of the causes of loss of expression.

1. 12. Transformation and Tissue Culture of *Hieracium aurantiacum*

Hieracium aurantiacum is routinely cultured *in vitro* (10). Organogenesis from shoot or leaf explant has previously been optimised in a basal medium (MS macro- and micro-salts, B5 vitamins, and 3% sucrose, solidified with 7.5% agar) supplemented with 0-4.9μM IBA and 0-17.8μM BA (10). Shoot formation is routinely obtained with 2.2μM BA and 2.5μM IBA (10). Shoots readily form roots in the absence of growth regulators. Although several attempts have been made to culture suspension cells and develop a regeneration protocol from single cells, no success has been achieved to date (Bicknell, Weld, pers. obs.). *A. tumefaciens*-mediated transformation of *H. aurantiacum* has been previously demonstrated (11). *H. aurantiacum* is susceptible to a range of antibiotics (eg, kanamycin, spectinomycin and streptomycin) facilitating the selection of stably transformed regenerants (11, Bicknell pers. comm.). There are no published procedures for direct gene delivery to *H. aurantiacum*.

H. aurantiacum is currently being used in our lab as a model plant for the study of apomixis. Gene tagging using a *Ds* element for insertional mutagenesis is being used to identify the gene(s) responsible for apomixis. *H. aurantiacum* is an easily transformed (by *A. tumefaciens*) plant that regenerates readily in tissue culture. For these reasons, I used *H. aurantiacum* as a model to test the components of the *Ac/Ds* transformation system to be used in onion. The *Ac/Ds* transposition system in *H. aurantiacum* was then used to study transient expression and further developed as a novel gene tagging strategy.

1. 13. Transformation and Tissue Culture of *Nicotiana plumbaginifolia*

Procedures for *N. plumbaginifolia* plant regeneration from cultured protoplasts were first developed for studies on somatic plant cell genetics in the 1970's (14). These procedures followed on from methods originally developed for *N. tabacum* culture (14). Since that time, *Nicotiana* protoplast systems have been widely used to study plant transformation (eg. 2, 70, 115, 131, 165). Consequently, there is a plethora of literature describing *Nicotiana* protoplast isolation and culture. However, the basic techniques used widely today have remained essentially unchanged from early procedures (eg. 14, 92, 100, 102, 109). Protoplasts are typically isolated from leaf mesophyll cells or cultured suspension cells by cell wall digestion. Protoplasts are washed and cultured at a typical density of around 10^4 protoplasts per ml. Culturing protoplasts at low density (around 100 cells per ml) without the use of feeder cells or a conditioned medium can be enhanced by using higher concentrations of 2, 4-D than typically used for high density growth (100). Where protoplasts are embedded at low density in the culture medium, solidification with agarose rather than agar has also been shown to improve initial cell division and sustained colony growth (92). *N. plumbaginifolia* suspension cell culture was chosen as the model system for fate mapping of transiently expressing cells because it was the availible plant system from which single transformed cells could most likely be isolated and cultured.

1. 14. References

1. Ambros PF, Matzke AJM, Matzke MA: Localisation of *Agrobacterium rhizogenes* T-DNA in plant chromosomes by *in situ* hybridisation. The EMBO Journal 5: 2073-2077 (1986).

2. An G: High efficiency transformation of cultured tobacco cells. Plant Physiology 79: 568-570 (1985).

3. Atkinson PW, Warren WD, O'Brochta DA: The *hobo* transposable element of *Drosophila* can be cross-mobilized in houseflies and excises like the *Ac* element of maize. Proceedings of the National Academy of Science USA 90: 9693-9697 (1993).

4. Bancroft I, Bhatt A M, Sjodin C, Scofield S, Jones JDG, Dean C: Development of an efficient two-element transposon tagging system in *Arabidopsis thaliana.* Molecular and General Genetics 233: 449-461 (1992).

5. Beaujean A, Sangwan RS, Hodges M, Sangwan-Norreel BS: Effect of ploidy and homozygosity on transgene expression in primary tobacco transformants and their androgenetic progenies. Molecular and General Genetics 260: 362-371 (1998).

6. Becker HA, Kunze R: *Ac* Transposase binds *in vitro* to the *Ac/Ds* terminal inverted repeats. Maize Genetics Corporation Newsletter 69: 38 (1995).

7. Beijersbergen A, Den Dulk-Ras A, Schilperoort RA, Hooykaas PJJ: Conjugative transfer by the virulence system of *Agrobacterium tumefaciens.* Science 256: 1324-1327 (1992).

8. Bellucci M, Alpini A, Paolocci F, Damiani F, Arcioni S: Transcription of a maize cDNA in *Lotus corniculatus* is regulated by T-DNA methylation and transgene copy number. Theoretical and Applied Genetics 98: 257-264 (1999).

9. Benediktsson I, Spampinato CP, Schieder O: Studies of the mechanism of transgene integration into plant protoplasts: improvement of the transformation rate. Euphytica 85: 53-61 (1995).

10. Bicknell RA: Micropropagation of *Hieracium aurantiacum.* Plant Cell, Tissue and Organ Culture 37: 197-199 (1994).

11. Bicknell RA, Borst NK: *Agrobacterium*-mediated transformation of *Hieracium aurantiacum.* International Journal of Plant Science 155(4): 467-470 (1994).

12. Binns AN: *Agrobacterium*-mediated gene delivery and the biology of host range limitations. Physiologia Plantarum 79: 135-139 (1990).

13. Bommineni VR, Jauhar PP: An evaluation of target cells and tissues used in genetic transformation of cereals. Maydica 42: 107-120 (1997).

14. Bougin J-P, Chupeau Y, Missonier C: Plant regeneration from mesophyll protoplasts of several *Nicotiana* species. Physiologia Plantarum 45: 288-292 (1979).

15. Brandes A, Heslop-Harrison JS, Kamm A, Kubis S, Doudrick RL, Schmidt T: Comparative analysis of the chromosomal and genomic organisation of *Ty1-copia*-like Retrotransposons in pteridophytes, gymnosperms and angiosperms. Plant Molecular Biology 33: 11-21 (1997).

16. Bundock P, den Dulk-Ras A, Beijersbergen A, Hooykaas PJJ: Trans-kingdom T-DNA transfer from *Agrobacterium tumefaciens* to *Saccharomyces cerevisiae*. The EMBO Journal 14: 3206-3214 (1995).

17. Cabrera-Ponce JL, Lopez L, Assad-Garcia N, Medina-Arevalo C, Bailey AM, Herrera-Estrella L: An efficient particle bombardment system for the genetic transformation of *Asparagus* (*Asparagus officinalis* L.). Plant Cell Reports 16: 255-260 (1997).

18. Calvi BR, Hong TJ, Findley SD, Gelbart WM: Evidence for a common evolutionary origin of inverted repeat transposons in *Drosophila* and Plants: *hobo*, *Activator*, and *Tam3*. Cell 66: 465-471 (1991).

19. Carels N, Barakat A, Barnardi G: The gene distribution of the maize genome. Proceedings of the National Academy of Science USA 92: 11057-11060 (1995).

20. Carrer H, Maliga P: Targeted insertion of foreign genes into the tobacco plastid genome without physical linkage to the selectable marker gene. Biotechnology 13: 791-794 (1995).

21. Carroll B, Klimyuk V, Thomas CM, Bishop GJ, Harrison K, Scofield S, Jones J: Germinal transpositions of the maize element *Dissociation* from T-DNA loci in tomato. Genetics 139: 407-420 (1995).

22. Chatterjee S, Starlinger P: The role of subterminal sites of transposable element *Ds* of *Zea mays* in excision. Molecular and General Genetics 249: 281-288 (1995).

23. Chen J, Greenblatt IM, Dellaporta SL: Molecular analysis of *Ac* transposition and DNA replication. Genetics 130: 665-676 (1992).

24. Chilton MD, Saiki RK, Yadav N, Gordon MP, Quetier F: T-DNA from *Agrobacterium* Ti plasmid is in the nuclear DNA fraction of crown gall tumour cells. Proceedings of the National Academy of Science USA 77: 4060-4064 (1980).

25. Chyi YS, Jorgensen RA, Goldstein D, Tanksley SD, Loaiza-Figueroa F: Locations and stability of *Agrobacterium*-mediated T-DNA insertions in the *Lycopersicon* genome. Molecular and General Genetics 204: 64-69 (1986).

26. Citovsky V, Guralnick B, Simon MN, Wall JS: The molecular structure of *Agrobacterium* VirE2-single-stranded DNA complexes involved in nuclear import. Journal of Molecular Biology 27: 718-727 (1997).

27. Citovsky V, Warnick D, Zambryski P: Nuclear import of *Agrobacterium* VirD2 and VirE2 proteins in maize and tobacco. Proceedings of the National Academy of Science USA 91: 3210-3214 (1994).

28. Citovski V, Zupan J, Warnick D, Zambryski P: Nuclear localisation of *Agrobacterium* VirE2 protein in plant cells. Science 256: 1802-1805 (1992).

29. Close TJ, Kado CI: High levels of double-stranded transferred DNA (T-DNA) processing from an intact nopaline Ti plasmid. Proceedings of the National Academy of Science USA 86: 2133-2137 (1989).

30. Close TJ, Tait RC, Kado CI: Regulation of Ti plasmid virulence genes by a chromosomal locus of *Agrobacterium tumefaciens*. Journal of Bacteriology 164: 774-781 (1985).

31. Cluster PD, O'Dell M, Metzlaff M, Flavell RB: Details of T-DNA structural organisation from a transgenic *Petunia* population exhibiting co-suppression. Plant Molecular Biology 32: 1197-1203 (1996).

32. Conner JA, Tanikanjana T, Stein JC, Kandasamy MK, Nasrallah JB, Nasrallah ME: Transgene-induced silencing of S-locus genes and related genes in *Brassica*. The Plant Journal 11: 809-823 (1997).

33. Daley M, Knauf VC, Summerfelt KR, Turner JC: Co-transformation with one *Agrobacterium tumefaciens* strain containing two binary plasmids as a method for producing marker-free transgenic plants. Plant Cell Reports 17: 489-496 (1998).

34. Davey MR, Rech EL, Mulligan BJ: Direct DNA transfer to plant cells. Plant Molecular Biology 13: 273-285 (1989).

35. De Buck S, Jacobs A, Van Montagu M, Depicker A: *Agrobacterium tumefaciens* transformation and cotransformation frequencies of *Arabidopsis thaliana* root explants and tobacco protoplasts. Molecular Plant-Microbe Interactions 11(6): 449-457 (1998).

36. de Kathen A, Jacobsen H-J: Cell competence for *Agrobacterium*-mediated DNA transfer in *Pisum sativum* L. Transgenic Research 4: 184-191 (1995).

37. Eady C: Towards the transformation of onions (*Allium cepa*). New Zealand Journal of Crop and Horticultural Science 23: 239-250 (1995).

38. Eady CC, Lister CE: A comparison of four selective agents for use with *Allium cepa* L. immature embryos and immature embryo-derived cultures. Plant Cell Reports 18: 117-121 (1998).

39. Eady CC, Lister CE, Suo Y, Schaper D: Transient expression of *uidA* constructs in *in vitro* onion (*Allium cepa* L.) cultures following particle bombardment and *Agrobacterium*-mediated DNA delivery. Plant Cell Reports 15: 958-962 (1996).

40. Eady CC, Weld RJ, Lister CE: *Agrobacterium tumefaciens*-mediated transformation and regeneration of onion (*Allium cepa* L.) plants. Plant Cell Reports (in press).

41. El-Kharbotly A, Jacobs JME, Hekkert B, Jacobsen E, Ramanna MS, Stiekema WJ, Pereira A: Localisation of *Ds*-transposon containing T-DNA inserts in the diploid transgenic potato: linkage to the R1 resistance gene against *Phytophthora infestans* (Mont.) de Bary. Genome 39: 249-257 (1996).

42. Elomaa P, Helariutta Y, Griesbach RJ, Kotilainen M, Sepanen P, Teeri TH: Transgene inactivation in *Petunia hybrida* is influenced by the properties of the foreign gene. Molecular and General Genetics 248: 649-656 (1995).

43. English JJ, Mueller E, Baulcombe DC: Suppression of virus accumulation in transgenic plants exhibiting silencing of nuclear genes. The Plant Cell 8: 179-188 (1996).

44. Essers L, Kunze R: Transposable elements *Bg* (*Zea mays*) and *Tag1* (*Arabidopsis thaliana*) encode protein sequences with homology to *Ac*-like transposases. Maize Genetics Corporation Newsletter 69: 39-41 (1995).

45. Essers L, Kunze R: Analysis of *Ac* transposase oligomerisation using the two hybrid system. Maize Genetics Corporation Newsletter 69: 41 (1995).

46. Essers L, Kunze R: Suppressor activity of *Ac* transposase in transfected *Petunia* cells. Maize Genetics Corporation Newsletter 69: 41 (1995).

47. Fedoroff NV: Maize transposable elements. In: Berg DE, Howe MM (eds) Mobile DNA, pp 375-411. American Society for Microbiology, Washington D.C. (1989).

48. Filichkin SA, Gelvin SB: Formation of a putative relaxation intermediate during T-DNA processing directed by the *Agrobacterium tumefaciens* VirD1, D2 endonuclease. Molecular Microbiology 8: 915-926 (1993).

49. Finnegan E J, Lawrence G J, Dennis E S, Ellis J G: Behaviour of modified *Ac* elements in flax callus and regenerated plants. Plant Molecular Biology 22: 625-633 (1993).

50. Fitzmaurice W P, Lehman L V, Thompson W F, Wernsman E A, Conkling M A: Development and characterization of a generalized gene tagging system for higher plants using an engineered maize transposon *Ac*. Plant Molecular Biology 20: 177-198 (1992).

51. Fridlender M, Harrison K, Jones JDG, Levy AA: Repression of the *Ac*-transposase gene promoter by *Ac* transposase. The Plant Journal 9: 911-917 (1996).

52. Gallo-Meagher M, Irvine JE: Effects of tissue type and promoter strength on transient GUS expression in sugarcane following particle bombardment. Plant Cell Reports 12: 666-670 (1993).

53. Geier T, Sangwan RS: Histology and chimeral segregation reveal cell-specific differences in the competence for shoot regeneration and *Agrobacterium*-mediated transformation in *Kohleria* internode explants. Plant Cell Reports 15: 386-390 (1996).

54. Gheysen G, Villarroel R, Van Montagu M: Illegitimate recombination in plants: a model for T-DNA integration. Genes & Development 5: 287-297 (1990).

55. Gleave AP, Mitra DS, Mudge SR, Morris BAM: Selectable marker-free transgenic plants without sexual crossing: transient expression of *cre* recombinase and use of a conditional lethal dominant gene. Plant Molecular Biology 40: 223-235 (1999).

56. Gray AJ, Raybould AF: Reducing transgene escape routes. Nature 392: 653-654 (1998).

57. Greenblatt IM, Brink RA: Twin mutations in medium variegated pericarp maize. Genetics 47: 489-501 (1961).

58. Handler AM, Gomez SP: The *hobo* transposable element excises and has related elements in Tephritid species. Genetics 143: 1339-1347 (1996).

59. Handler AM, Gomez SP: The *hobo* transposable element has transposase-dependent and -independent excision activity in drosophilid species. Molecular and General Genetics 247: 399-408 (1995).

60. Hansen G, Chilton M-D: Agrolistic transformation of plant cells: integration of T-strands generated *in planta*. Proceedings of the National Academy of Science USA 93: 14978-14983 (1996).

61. Haseloff J, Siemering KR, Prasher DC, Hodge S: Removal of a cryptic intron and subcellular localisation of green fluorescent protein are required to mark transgenic *Arabidopsis* plants brightly. Proceedings of the National Academy of Sciences USA 94: 2122-2127 (1997).

62. Heinemann JA: Genetic evidence of protein transfer during bacterial conjugation. Plasmid 41: 240-247 (1999).

63. Heinemann JA: Genetics of gene transfer between species. Trends in Genetics 7 (6): 181-185. (1991).

64. Heinlein M: Excision patterns of *Activator* (*Ac*) and *Dissociation* (*Ds*) elements in *Zea mays* L: implications for the regulation of transposition. Genetics 144: 1851-1869 (1996).

65. Herman L, Jacobs A, Van Montagu M, Depicker A: Plant chromosome/marker gene fusion assay for study of normal and truncated T-DNA integration events. Molecular and General Genetics 224: 248-256 (1990).

66. Herrera-Estrella A, Chen Z, Van Montagu M, Wang K: VirD proteins of *Agrobacterium tumefaciens* are required for the formation of a covalent DNA-protein complex at the 5' terminus of the T-strand molecules. The EMBO Journal 7: 4055-4062 (1988).

67. Hooykaas PJJ, Hofker M, den Dulk-Ras H, Schilperoort RA: A comparison of virulence determinants in an octopine Ti plasmid, a nopaline Ti plasmid, and an Ri plasmid by complementation analysis of *Agrobacterium tumefaciens* mutants. Plasmid 11: 195-205 (1984).

68. Horsch RB, Fry JE, Hoffman NL, Wallroth M, Eicholtz D, Rogers SG, Fraley RT: A simple and general method for transferring genes into plants. Science 227: 1229-1231 (1985).

69. Houba-Herin N, Domin M, Leprince A-S: Some features about transposition of the maize element *Dissociation* in *Nicotiana plumbaginifolia*. Genetica 93: 41-48 (1994).

70. Houba-Herin N, Domin M, Pedron J: Transposition of a *Ds* element from a plasmid into the plant genome in *Nicotiana plumbaginifolia* protoplast-derived cells. The Plant Journal 6: 55-66 (1994).

71. Hunold R, Bronner R, Hahne G: Early events in microprojectile bombardment: cell viability and particle location. The Plant Journal 5: 593-604 (1994).

72. Janssen B-J, Gardner RC: Localized transient expression of GUS in leaf discs following cocultivation with *Agrobacterium*. Plant Molecular Biology 14: 61-72 (1989).

73. Jarchow E, Grimsley NH, Hohn B: *vir*F, the host-range-determining virulence gene of *Agrobacterium tumefaciens*, affects T-DNA transfer to *Zea mays*. Proceedings of the National Academy of Science USA 88: 10426-10430 (1991).

74. Jones JDG, Carland F, Lim E, Ralston E, Dooner HK: Preferential transposition of the maize element *Activator* to linked chromosomal locations in tobacco. Plant Cell 2: 701-707 (1990).

75. Jorgensen RA, Cluster PD, English J, Que Q, Napoli CA: Chalcone synthase cosuppression phenotypes in petunia flowers: comparison of sense vs. antisense constructs and single copy vs. complex T-DNA sequences. Plant Molecular Biology 31: 957-973 (1996).

76. Kass SU, Pruss D, Wolffe AP: How does DNA methylation repress transcription? Trends in Genetics 13: 444-449 (1997).

77. Keller J, Lim E, Dooner H K: Preferential transposition of *Ac* to linked sites in *Arabidopsis*. Theoretical and Applied Genetics 86: 585-588 (1993).

78. Kohler F, Cardon G, Pohlman M, Gill R, Schieder O: Enhancement of transformation rates in higher plants by low-dose irradiation: are DNA repair systems involved in the incorporation of exogenous DNA into the plant genome? Plant Molecular Biology 12: 189-199 (1989).

79. Koncz C, Martin N, Mayerhofer R, Koncz-Kalman Z, Korber H, Redei GP, Schell J: High-frequency T-DNA-mediated gene tagging in plants. Proceedings of the National Academy of Science USA 86: 8467-8471 (1989).

80. Kost B, Galli A, Potrykus I, Neuhaus G: High efficiency transient and stable transformation by optimized DNA microinjection into *Nicotiana tabacum* protoplasts. Journal of Experimental Botany 46(290): 1157-1167 (1991).

81. Koukolikova-Nicola Z, Shillito RD, Hohn B, Wang K, Montagu MV, Zambryski P: Involvement of circular intermediates in the transfer of T-DNA from *Agrobacterium tumefaciens* to plant cells. Nature 313: 191-196 (1985).

82. Kumpatia SP, Chandrasekharan MB, Iyer LM, Li G, Hall TC: Genome intruder scanning and modulation systems and transgene silencing. Trends in Plant Science 3: 97-104 (1998).

83. Kunze R: The maize transposable element *Activator* (*Ac*). In: Current Topics in Microbiology and Immunology, Vol. 204. Springer-Verlag, Berlin, New York, pp. 161-194.

84. Laufs J, Wirtz U, Kammann M, Matzeit V, Schaefer S, Schell J, Czernilofsky AP, Baker B, Gronenborn B: Wheat Dwarf Virus *Ac/Ds* vectors: expression and excision of transposable elements introduced into various cereals by a viral replicon. Proceedings of the National Academy of Sciences USA 87: 7752-7756 (1990).

85. Lebel EG, Masson J, Bogucki A, Paszkowski J: Transposable elements as plant transformation vectors for long stretches of foreign DNA. Theoretical and Applied Genetics 91: 899-906 (1995).

86. Lee KY, Lund P, Lowe K, Dunsmuir P: Homologous recombination in plant cells after *Agrobacterium*-mediated transformation. Plant Cell 2: 415-425 (1990).

87. Lee Y-W, Jin S, Sim W-S, Nester EW: Genetic evidence for direct sensing of phenolic compounds by the *vir*A protein of *Agrobacterium tumefaciens*. Proceedings of the National Academy of Science USA 92: 12245-12249 (1995).

88. Lessl M, Lanka E: Common mechanisms in bacterial conjugation and Ti-mediated T-DNA transfer to plant cells. Cell 77: 321-324 (1994).

89. Levy AA, Fridlender M, Hanania U, Rubin E, Sitrit Y: Binding of *Nicotiana* nuclear proteins to the subterminal regions of the *Ac* transposable element. Molecular and General Genetics 251: 436-441 (1996).

90. Lisch D, Chomet P, Freeling M: Genetic characterization of the *Mutator* system in maize: behaviour and regulation of *Mu* transposons in a minimal line. Genetics 139: 1777-1796 (1995).

91. Livingstone DM, Birch RG: Plant regeneration and microprojectile-mediated gene transfer in embryonic leaflets of peanut (*Arachis hypogaea* L.). Australian Journal of Plant Physiology 22: 585-591 (1995).

92. Lorz H, Larkin PJ, Thomson J, Scowcroft WR: Improved protoplast culture and agarose media. Plant Cell, Tissue and Organ Culture 2: 217-226 (1983).

93. Martienssen RA, Richards EJ: DNA methylation in eukaryotes. Current Opinion in Genetic Development 5: 234-242 (1995).

94. Marton L, Hrouda M, Pecsvaradi A, Czako M: T-DNA-insert-independent mutations induced in transformed plant cells during *Agrobacterium* co-cultivation. Transgenic Research 3: 317-325 (1994).

95. Matsumoto S, Ito Y, Hosoi T, Takahashi Y, Machida Y: Integration of *Agrobacterium* T-DNA into a tabacco chromosome: possible involvement of DNA homology between T-DNA and plant DNA. Molecular and General Genetics 224: 309-316 (1990).

96. Matzke MA, Matzke AJ: How and why do plants inactivate homologous (trans)genes? Plant Physiology 107: 679-685 (1995).

97. Mayerhofer R, Koncz-kalman Z, Nawrath C, Bakkeren G, Crameri A, Angelis K, Redei GP, Schell J, Hohn B, Koncz C: T-DNA integration: a mode of illegitimate recombination in plants. The EMBO Journal 10(3): 697-704 (1991).

98. Meyer P, Walgenbach E, Bussmann K, Hombrecher G, Saedler H: Synchronized tobacco protoplasts are efficiently transformed by DNA. Molecular and General Genetics 201: 513-518 (1985).

99. Mozo T, Hooykaas PJJ: Factors affecting the rate of T-DNA transfer from *Agrobacterium tumefaciens* to *Nicotiana glauca* plant cells. Plant Molecular Biology 19: 1019-1030 (1992).

100. Muller JF, Missionier C, Caboche M: Low density growth of cells derived from *Nicotiana* and *Petunia* protoplasts: influence of the source of protoplasts and comparison of the growth-promoting activity of various auxins. Physiologia Plantarum 57: 35-41 (1983).

101. Mysore KS, Bassuner B, Deng X, Darbinian NS, Motchoulski A, Ream W, Gelvin SB: Role of the *Agrobacterium tumefaciens* VirD2 protein in T-DNA transfer and integration. Molecular Plant-Microbe Interactions 11(7): 668-683 (1998).

102. Nagy JI, Maliga P: Callus induction and plant regeneration from mesophyll protoplasts of *Nicotiana sylvestris*. Z Pflanzenphysiol Bd 78: 453-455 (1976).

103. Nam J, Matthysse AG, Gelvin SB: Differences in susceptibility of *Arabidopsis* ecotypes to crown gall disease may result from a deficiency in T-DNA integration. The Plant Cell 9: 317-333 (1997).

104. Nam J, Mysore KS, Gelvin SB: *Agrobacterium tumefaciens* transformation of the radiation hypersensitive *Arabidopsis thaliana* mutants *uvh*1 and *rad*5. Molecular Plant-Microbe Interactions 11(11): 1136-1141 (1998).

105. Nam J, Mysore KS, Zheng C, Knue MK, Matthysse AG, Gelvin SB: Identification of T-DNA tagged *Arabidopsis* mutants that are resistant to transformation by *Agrobacterium*. Molecular and General Genetics 261: 429-438 (1999).

106. Nandadeva YL, Lupi CG, Meyer CS, Devi PS, Potrykus I, Bilang R: Microprojectile-mediated transient and integrative transformation of rice embryogenic suspension cells: effects of osmotic cell conditioning and of physical configuration of plasmid DNA. Plant Cell Reports 18: 500-504 (1999).

107. Narasimhulu SB, Deng X, Sarria R, Gelvin SB: Early transcription of *Agrobacterium* T-DNA genes in tobacco and maize. The Plant Cell 8: 873-886 (1996).

108. Narayan R, Hinton H, Walter C: Development of a novel transformation technique for plant cells involving *Agrobacterium tumefaciens* Ti-Plasmid border sequences, *vir*D and *vir*E gene products and biolistics. Abstracts of the New Zealand Branch of the IAPTC Conference 1997: 49 (1997).

109. Negrutiu I, De Brouwer D, Watts JW, Sidorov VI, Dirks R, Jacobs M: Fusion of plant protoplasts: a study using auxotrophic mutants of *Nicotiana plumbaginifolia*, Viviani. Theoretical and Applied Genetics 72: 279-286 (1986).

110. Palauqui JC, Balzergue S: Activation of systemic silencing by localised introduction of DNA. Current Biology 9: 59-66 (1999).

111. Palauqui JC, Vaucheret H: Transgenes are dispensable for the RNA degradation step of cosuppression. Proceedings of the National Academy of Science USA 95: 9675-9680 (1998).

112. Palmgren G, Mattson O, Okkels F: Treatment of *Agrobacterium* or leaf disks with 5-azacytidine increases transgene expression in tobacco. Plant Molecular Biology 21: 429-435 (1993).

113. Pan SQ, Charles T, Jin S, Wu Z-L, Nester EW: Preformed dimeric state of the sensor protein VirA is involved in plant-*Agrobacterium* signal transduction. Proceedings of the National Academy of Science USA 90: 9939-9943 (1993).

114. Park YD, Papp I, Moscone EA, Iglesias VA, Vaucheret H, Matzke AJM, Matzke MA: Gene silencing mediated by promoter homology occurs at the level of transcription and results in meiotically heritable alterations in methylation and gene activity. The Plant Journal 9: 183-194 (1996).

115. Paszkowski J, Shillito RD, Saul M, Mandak V, Hohn B, Potrykus I: Direct gene transfer to plants. The EMBO Journal 3(12): 2717-2722 (1984).

116. Potrykus I: Gene transfer to plants: assessment and perspectives. Physiologia Plantarum 79: 125-134 (1990).

117. Preuss SB, Jiang CZ, Baik HK, Kado CI, Britt AB: Radiation-sensitive *Arabidopsis* mutants are proficient for T-DNA transformation. Molecular and General Genetics 261: 623-626 (1999).

118. Prols F, Meyer P: The methylation patterns of chromosomal integration regions influence gene activity of transferred DNA in *Petunia hybrida*. The Plant Journal 2: 465-475 (1992).

119. Prols M, Topfer R, Scheh J, Steinbiss HH: Transient gene expression in tobacco protoplasts: Time course of CAT appearence. Plant Cell Reports 7: 221-224 (1988).

120. Ratcliff F, Harrison BD, Baulcombe DC: A similarity between viral defense and genome silencing in plants. Science 276: 1558-1560 (1997).

121. Regensburg-Tuink AJG, Hooykaas PJJ: Transgenic *N. glauca* plants expressing bacterial virulence gene *vir*F are converted into hosts for nopaline strains of *A. tumefaciens*. Nature 363: 69-70 (1993).

122. Risseeuw E, Franke-van Dijk M, Hooykaas PJJ: Gene targeting and instability of *Agrobacterium* T-DNA loci in the plant genome. The Plant Journal 11: 717-728 (1997).

123. Ronchi A, Petroni K, Tonelli C: The reduced expression of endogenous duplications (REED) in the maize R gene family is mediated by DNA methylation. The EMBO Journal 14(21): 5318-5328 (1995).

124. Rossi L, Hohn B, Tinland B: Integration of complete transferred DNA units is dependent on the activity of virulence E2 protein of *Agrobacterium tumefaciens*. Proceedings of the National Academy of Science USA 93: 126-130 (1996).

125. Rossi L, Hohn B, Tinland B: The VirD2 protein of *Agrobacterium tumefaciens* carries nuclear localization signals important for transfer of T-DNA to plants. Molecular and General Genetics 239: 345-353 (1993).

126. Ruiz MT, Voinnet O, Baulcombe DC: Initiation and maintenance of virus-induced gene silencing. The Plant Cell 10: 937-946 (1998).

127. Sabl J F, Henikoff S: Copy number and orientation determine the susceptibility of a gene to silencing by nearby heterochromatin in *Drosophila*. Genetics 142: 447-458 (1996).

128. Scofield SR, Harrison K, Nurrish SJ, Jones JDG: Promoter fusions to the *Activator* transposase gene cause distinct patterns of *Dissociation* excision in tobacco cotyledons. The Plant Cell 4: 573-582 (1992).

129. Scott L, LaFoe D, Weil CF: Adjacent sequences influence DNA repair accompanying transposon excision in maize. Genetics 142: 237-246 (1996).

130. Shen W-H, Hohn B: Vectors based on maize streak virus can replicate to high copy numbers in maize plants. Journal of General Virology 76: 965-969 (1995).

131. Shillito R, Saul M, Paszkowski J, Muller M, Potrykus I: High efficiency direct gene transfer to plants. Biotechnology 3: 1099-1103 (1985).

132. Shimamoto K, Miyazaki C, Hashimoto H, Izawa T, Itoh K, Terada R, Inagaki Y, Iida S: *Trans*-activation and stable integration of the maize transposable element *Ds* cotransfected with the *Ac* transposase gene in transgenic rice plants. Molecular and General Genetics 239: 354-360 (1993).

133. Songstad DD, Halaka FG, DeBoer DL, Armstrong CL, Hinchee MAW, Ford-Santino CG, Brown SM, Fromm ME, Horsch RB: Transient expression of GUS and anthocyanin constructs in intact maize immature embryos following electroporation. Plant Cell, Tissue and Organ Culture 33: 195-201 (1993).

134. Songstad DD, Somers DA, Griesbach RJ: Advances in alternative DNA delivery techniques. Plant Cell, Tissue and Organ Culture 40: 1-15 (1995).

135. Sonti RV, Chiurazzi M, Wong D, Davies CS, Harlow GR, Mount DW, Signer ER: *Arabidopsis* mutants deficient in T-DNA integration. Proceedings of the National Academy of Science 92: 11786-11790 (1995).

136. Stachel SE, Nester EW: The genetic and transcriptional organisation of the *vir* region of the Ti plasmid of *Agrobacterium tumefaciens*. The EMBO Journal 5: 1445-1454 (1986).

137. Stachel SE, Timmerman B, Zambryski P: Activation of *Agrobacterium tumefaciens vir* gene expression generates multiple single-stranded T-strand molecules from the pTiA6 T-region: requirement for 5' *vir*D gene products. The EMBO Journal 6: 857-863 (1987).

138. Stachel SE, Timmerman B, Zambryski P: Generation of single-stranded T-DNA molecule during the initial stages of T-DNA transfer from *Agrobacterium tumefaciens* to plant cells. Nature 322: 706-712 (1986).

139. Stachel SE, Zambryski PL: *Agrobacterium tumefaciens* and the susceptible plant cell: a novel adaptation of extracellular recognition and DNA conjugation. Cell 47: 155-157 (1986).

140. Stam M, Mol JNM, Kooter JM: The silence of genes in transgenic plants. Annals of Botany 79: 3-12 (1997).

141. Suarez-Lopez P, Gutierrez C: DNA replication of wheat dwarf geminivirus vectors: effects of origin structure and size. Virology 227: 389-399 (1997).

142. Sugimoto K, Otsuki Y, Saji S, Hirochika H: Transposition of the maize *Ds* element from a viral vector to the rice genome. The Plant Journal 5: 863-871 (1994).

143. Swinburne J, Balcells L, Scofield SR, Jones JDG, Coupland G: Elevated levels of *Activator* transposase mRNA are associated with high frequencies of *Dissociation* excision in *Arabidopsis*. The Plant Cell 4: 583-595 (1992).

144. Thierry D, Vaucheret H: Sequence homology requirements for transcriptional silencing of 35S transgenes and post-transcriptional silencing of nitrite reductase (trans)genes by the tobacco 271 locus. Plant Molecular Biology 32: 1075-1083 (1996).

145. Timmermans MCP, Das OP, Messing J: *Trans*-replication and high copy numbers of wheat dwarf virus vectors in maize cells. Nucleic Acids Research 20(15): 4047-4054 (1992).

146. Tindland B, Hohn B, Puchta H: *Agrobacterium tumefaciens* transfers single-stranded transferred DNA (T-DNA) into the plant cell nucleus. Proceedings of the National Academy of Science USA 91: 8000-8004 (1994).

147. Tindland B, Schoumacher F, Gloeckler V, Bravo-Angel AM, Hohn B: The *Agrobacterium tumefaciens virulence* D2 protein is responsible for precise integration of T-DNA into the plant genome. The EMBO Journal 14(14): 3585-3595 (1995).

148. Ugaki M, Ueda T, Timmermans M, Vieira J, O.Elliston K, Messing J: Replication of a geminivirus derived shuttle vector in maize endosperm cells. Nucleic Acids Research 19(2): 371-377 (1991).

149. Vain P, McMullen MD, Finer JJ: Osmotic treatment enhances particle bombardment-mediated transient and stable transformation of maize. Plant Cell Reports 12: 84-88 (1993).

150. Vergunst AC, Hooykaas PJJ: *Cre/lox*-mediated site-specific integration of *Agrobacterium* T-DNA in *Arabidopsis thaliana* by transient expression of *cre*. Plant Molecular Biology 38: 393-406 (1998).

151. Vergunst AC, Hooykaas PJJ: Recombination in the plant genome and its application in biotechnology. Critical Reviews in Plant Sciences 18(1): 1-31 (1999).

152. Villemont E, Dubois F, Sangwan RS, Vasseur G, Bourgeois Y, Sangwan-Norreel BS: Role of the host cell cycle in the *Agrobacterium*-mediated genetic transformation of *Petunia*: evidence of an S-phase control mechanism for T-DNA transfer. Planta 201: 160-172 (1997).

153. Voinnet O, Vain P, Angell S, Baulcombe DC: Systemic spread of sequence-specific transgene RNA degradation in plants is initiated by localised introduction of ectopic promoterless DNA. Cell 95: 177-187 (1998).

154. Walden DB, Vetsch CS, Bommineni VR, Pareddy DR, Sanchez JP, Banasikowska E, Kudirka DT: Maize meristem culture and recovery of mature plants. Maydica 34: 263-275 (1989).

155. Wang K, Genetello C, Van Montagu M, Zambryski P: Sequence context of the T-DNA border repeat element determines its relative activity during T-DNA transfer to plant cells. Molecular and General Genetics 210: 338-346 (1987).

156. Wang L, Heinlein M, Kunze R: Methylation pattern of *Activator* transposase binding sites in maize endosperm. The Plant Cell 8: 747-758 (1996).

157. Wang L, Heinlein M, Starlinger P, Kunze R: Analysis of the methylation state of the transposase binding sites in the termini of *Ac*. Maize Genetics Corporation Newsletter 69: 39 (1995).

158. Wang K, Herrera-Estrella L, Van Montagu M, Zambryski P: Right 25bp terminus sequences of the nopaline T-DNA is essential for and determines direction of DNA transfer from *Agrobacterium* to the plant genome. Cell 38: 455-462 (1984).

159. Ward E, Barnes W: VirD2 protein of *Agrobacterium tumefaciens* very tightly linked to the 5' end of T-strand. Science 242: 927-930 (1988).

160. Wassenegger M, Pelissier T: Signalling in gene silencing. Trends in Plant Science 4: 207-208 (1999).

161. Werr W, Lorz A: Transient gene expression in a *Gramineae* cell line. A rapid procedure for studying plant promoters. Molecular and General Genetics 202: 471-475 (1986).

162. Wirtz U, Osborne B, Baker B: *Ds* excision from extrachromosomal geminivirus vector DNA is coupled to vector DNA replication in maize. The Plant Journal 11(1): 125-135 (1997).

163. Woolston CJ, Reynolds HV, Stacey NJ, Mullineaux PM: Replication of wheat dwarf virus DNA in protoplasts and analysis of coat protein mutants in protoplasts and plants. Nucleic Acids Research 17(15): 6029-6041 (1989).

164. Wu FS, Feng TY: Delivery of plasmid DNA into intact plant cells by electroporation of plasmolyzed cells. Plant Cell Reports 18: 381-386 (1999).

165. Wullems GJ, Molendijk L, Ooms G, Schilperoort RA: Differential expression of crown gall tumor markers in transformants obtained after *in vitro* *Agrobacterium tumefaciens*-induced transformation of cell wall regenerating protoplasts derived from *Nicotiana tabacum*. Proceedings of the National Academy of Science USA 78: 4344-4348 (1981).

166. Yang CH, Carroll B, Scofield S, Jones J, Michelmore R: Transactivation of *Ds* elements in plants of lettuce (*Lactuca sativa*). Molecular and General Genetics 241: 389-398 (1993).

167. Ye F, Signer ER: RIGS (repeat-induced gene silencing) in *Arabidopsis* is transcriptional and alters chromatin configuration. Proceedings of the National Academy of Science USA 93: 10881-10886 (1996).

168. Yoshioka Y, Takahashi Y, Matsuoka K, Nakamura K, Koizumi J, Kojima M, Machida Y: Transient gene expression in plant cells mediated by *Agrobacterium tumefaciens*: application for the analysis of virulence loci. Plant Cell Physiology 37(6): 782-789 (1996).

169. Young C, Nester EW: Association of the VirD2 protein with the 5' end of T-strands in *Agrobacterium tumefaciens*. Journal of Bacteriology 170: 3367-3374 (1988).

170. Yusibov VM, Steck TR, Gupta V, Gelvin S: Association of single-stranded transferred DNA from *Agrobacterium tumefaciens* with tobacco cells. Proceedings of the National Academy of Science USA 91: 2994-2998 (1994).

171. Zambryski P: Chronicles from the *Agrobacterium*-plant cell DNA transfer story. Annual Review of Plant Physiology and Plant Molecular Biology 43: 465-490 (1992).

172. Ziemienowicz A, Gorlich D, Lanka E, Hohn B, Rossi L: Import of DNA into mammalian nuclei by proteins originating from a plant pathogenic bacterium. Proceedings of the National Academy of Science USA 96: 3729-3733 (1999).

173. Zupan J, Zambryski P: The *Agrobacterium* DNA transfer complex. Critical Reviews in Plant Sciences 16(3): 279-295 (1997).

Chapter 2.

Dissociation (*Ds*) Elements as Vectors for Transformation of Onion (*A. cepa*).

2. 1. Introduction.

Transformation of the important crop vegetable onion (*Allium cepa*) has been researched in this laboratory over several years. Embryogenic cell cultures derived from immature embryos have been shown to exhibit a high frequency of regeneration suitable for transformation (1, 2). Particle bombardment and *A. tumefaciens*-mediated DNA delivery have been optimised for high frequencies of transient expression (5). Hygromycin, geneticin and Basta have been identified as effective selective agents (4). Very recently, positive selection using the reporter gene *m-gfp5-ER* allowed the development of a repeatable, cultivar independent, onion transformation protocol (6).

Gene delivery to onion has resulted in a high frequency of transient expression and a frequency of stable expression several orders of magnitude lower. It has been suggested that the low frequency of stable expression could be increased if the transgene was carried on a transposable element. In such a system, the transgene (within the borders of a non-autonomous transposable element) would be delivered to the plant cell on plasmid DNA. The *trans*-acting functions necessary for transposition would be carried on a separate region of the plasmid or co-delivered simultaneously on a separate plasmid vector. Transient expression of the *trans*-acting functions in the plant cell would mediate transgene integration into the plant genome by transposition. This strategy assumes that transgene integration is a significant barrier to stable transformation in onion and that transposition would overcome that barrier.

Research supporting the above strategy is outlined below. Transgene integration, mediated by transposition of *Ac/Ds* elements, has previously been developed as both a transformation strategy (14, 15) and as a gene tagging strategy (10, 11, 18). *Ac/Ds* transposition from extrachromosomal plasmid DNA, catalysed by transiently expressed transposase, has been demonstrated in both dicotyledonous plants (tobacco: 10, 11, 15) and monocotyledonous plants (rice: 18, 19, wheat: 14, maize: 20). *Ds* elements have been shown to be capable of efficiently integrating inserts as large as 9.5kb (15). One group has reported a four-fold increase in recovery of stable transformants using *Ds*-mediated

integration (15). However, another group found no increase in the frequency of stable transformation using *Ds*-mediated integration (19).

In this chapter, initial research into transient expression and *Ds* transposition in onion is described. I sought to examine the phenomenon of transient expression in onion callus tissue and test whether loss of expression was due to cell death. Further, I sought to test the feasibility of using *Ds* transposition to integrate transgenes into the onion genome (Figure 1.1.1). To test *Ds* transposition in onion, transient expression of the *Ac* transposase gene was used to cause transposition of a *Ds* element from a plasmid vector after delivery to onion tissue by particle bombardment. The results presented here suggest that transient transposase expression can mediate *Ds* transposition in onion cells. It also appears that, as in other systems (10, 11) transposition is more frequent when a transposase gene lacking part of the untranslated leader sequence, rather than the full length gene, is used.

2. 2. Materials and Methods.

2. 2. 1. Plasmids.

pNT103

pNT103 (10) contains a *Ds* element inserted between the 1' promoter and a *uid*A gene. *Ds* excision allows expression of *uid*A (encodes β-glucuronidase). The *Ds* element carries a kanamycin resistance gene for selection in plant cells.

pNT[BamHI]

pNT[BamHI] is *pNT103* digested with *Bam*HI and religated to create a plasmid lacking the *Ds* element with the 1' promoter directly upstream from the *uid*A gene.

pSLJ1101

pSLJ1101 (13) contains a 3.7kb (*Pst*I-*Nae*I) fragment of *Ac* ligated to a tobacco mosaic virus 5' omega leader sequence downstream from the CaMV35S promoter. This fragment encodes *Ac* transposase ORFa translated from ATG1, ATG2 and ATG3.

pSLJ1101[EcoRI]

pSLJ1101[EcoRI] is *pSLJ1101* digested with *Eco*RI removing part of the transposase gene and the CaMV35S promoter and then recircularised by ligation.

pBINm-gfp5-ER

pBINm-gfp5-ER (9) is a binary vector containing the gene *m-gfp5-ER* (*gfp* gene modified for high expression in plants and encoding a GFP protein that is targeted to the plant cell endoplasmic reticulum to reduce any toxic effects) downstream from the CaMV35S promoter.

pNT804

pNT804 (11) contains an *Ac* transposase gene construct consisting of an *Ac* genomic fragment downstream of the 2' promoter of the octopine T-DNA. This truncated transposase fragment lacks ORFa ATG1 and ATG2 translation start sites and induces a higher excision frequency than the entire ORFa transposase gene in some plant systems (10).

pART8

pART8 is *pART7* (7) with the *uid*A gene inserted at the multi-cloning site (*Xho*I-*Bam*HI) downstream of the CaMV 35S promoter.

pRJW28

pRJW28 contains a *Ds* element interrupting the expression of a *uid*A marker gene. The *Ds* element carries a CaMV35S-multi cloning site-ocs expression cassette. To construct *pRJW28* a DNA fragment containing a *Ds* element was amplified by polymerase chain reaction (PCR) (primers: 5'-CGG TCG ACG GAT CCG CGC GGA GGG GGA G-3' and 5-CGG TCG ACC CCC GAC TCT AGA GGA TCC GC-3') from *pMDSBAR* (15). This fragment was digested with *Sal*I and inserted into the *Xho*I site of *pART8* creating *pRJW14*. *pRJW14* thus has the *Ds* element inserted between the CaMV35S promoter and the *uid*A marker gene. A DNA fragment containing the CaMV35s promoter-multi cloning site-OCS terminator cassette from *pART7* (7) flanked by *Sal*I restriction sites was amplified by PCR (primers: 5'-ACG CGT CGA CAC TAT AGA ATA TGC ATC A -3' and 5'- ACG CGT

CGA CGA ATT AAT TCC AAT CCC A-3'). This fragment was digested with *Sal*I and inserted into the *Xho*I site within the *Ds* element of *pRJW14* creating *pRJW28.*

2. 2. 2. Tissue culture

Allium cepa cell cultures were initiated and maintained from Canterbury Long Keeper onion immature embryos on EIM medium supplemented with 5mg/l picloram (P5 medium) as previously described (2).

2. 2. 3. Plasmid construction.

Preparation and transformation of competent *Escherichia coli* with plasmid DNA was by the heat shock method as previously described (16). Plasmid DNA was maintained and multiplied in *E. coli* DH5α (37^0C, 12 hours, shaking culture in LB medium) and extracted by standard alkaline lysis and phenol/chloroform extraction (16). Restriction enzymes were used as per manufacturer's (Boehringer Mannheim) instructions. For ligation (over night at room temperature) using T4 DNA ligase (New England Biolabs) manufacturer's instructions were essentially followed. Where fragments were inserted the vector was de-phosphorylated using shrimp alkaline phosphatase as per manufacturer's (Boehringer Mannhein) instructions. For vector construction, inserts were amplified by PCR performed in a Techne Thermocycler PHC-3 with 0.625 units *Taq* polymerase (Boehringer Mannheim) in a total volume of 25μl. Inserts were isolated by electrophoresis on a 0.7% agarose gel and extracted with a Qiagen gel extraction kit.

2. 2. 4 Bombardment conditions

All bombardments were with a Biolistic PDS-1000/He particle delivery system (Biorad). Plasmid DNA was adsorbed to gold particles (1-3μm diameter) by $CaCl_2$ (0.96M) and spermidine (0.015M) precipitation. The tissue was bombarded in a vacuum (25 inches of mercury) at a target distance (stop grid to leaf explant) of 8cm, and a rupture disk pressure of 1100-1300 psi.

2. 2. 5. *Ds* excision assay

Cultured onion cells were co-bombarded with a plasmid (*pNT103*) containing a *Ds* element interrupting expression of the marker gene *uid*A and a plasmid encoding the *Ac* transposase (either *pNT804* or *pSLJ1101*). In order to ensure independence between replicates, DNA was precipitated onto gold particles separately for each bombardment. For each replicate, a fixed area (200mm^2) of onion tissue was placed centrally on a petri dish containing P5 medium. Dishes were assigned to treatment at random. After bombardment, tissue was incubated in the dark at 22-26^{0}C for 5 days. All the tissue was stained for β-glucuronidase activity (colourless substrate is converted to a visible blue dye) and the total number of blue spots were counted under a stereo-microscope. Blue spots were counted blind (ie. the researcher was unaware which treatment had been applied to the tissue being observed).

2. 2. 6. Histochemical assay for β-glucuronidase activity.

uidA expression was monitored by histochemical localisation of β-glucuronidase activity by incubating plant tissue in 0.1M phosphate buffer containing: 1mM 5-bromo-4-chloro-3-indolyl β-D-glucuronide (x-glu); 2.5mM potassium ferricyanide and 2.5mM ferrocyanide (3). Cleavage of x-glu by β-glucuronidase results in a highly visible blue colour.

2. 3. Results.

2. 3. 1. Transient expression in onion cells.

Previous reports suggested that following gene transfer to onion tissue, a high frequency of transient expression could be observed (5). However, stable expression has been only infrequently observed. Confirmation of these observations was initially sought in the tissue culture and transformation system to be used (onion cell culture and particle bombardment). For examination of transient expression, and in order to demonstrate that *Ds* transposition could improve the ratio of transient to stable expression, a marker gene

was sought that would allow the accurate quantitation of transient and stable expression in the same tissue.

The *uid*A gene, with histochemical staining for β-glucuronidase activity, was a highly visible marker of transgene expression. However, histochemical staining was not cell-specific, leading to uncertainty in the frequency of expression. Also, the histochemical assay killed the onion tissue making it impossible to follow the fate of expressing cells. Expression of the maize anthocyanin regulatory gene *Lc* caused enhanced anthocyanin accumulation in some plants without affecting cell viability (8). *pAL144* (*Lc* downstream from the CaMV35S promoter) was bombarded into onion cell cultures which were monitored over several weeks. However, there was no discernable difference in visible anthocyanin accumulation between tissue bombarded with *pAL144* and tissue bombarded with *pART8* (negative control). *pBINm-gfp5-ER* (encoding the fluorescent protein GFP) was also bombarded into onion cell cultures. GFP expression was highly visible in onion cells. Further, GFP did not diffuse between cells and, as the GFP protein was targeted to the endoplasmic reticulum, the visible activity was very distinctive.

To examine transient expression in onion, *pBINm-gfp5-ER* was bombarded into cultured cells (organised growth of the types "embryogenic callus" and "compact callus" (2)). Two days after bombardment, in a sample of tissue from three bombardments, a total of 501 cells had visible GFP activity (GFP positive). In the same tissue, three days after bombardment, the number of GFP positive cells had fallen to 359. The number of GFP positive cells remained constant for the next 2 days and then steadily declined. There were 71 GFP positive cells by 2 weeks post bombardment and 30 GFP positive cells by 3 weeks. This pattern of expression was the same in tissue from all three bombardments and no stable expression was observed.

Observing the GFP activity involved directing blue light at the cells. This treatment can visibly wilt plant tissue and kill plant cells. To test whether repeatedly observing the cells under blue light had a major detrimental effect, a third of the sampled tissue was set aside after the first observation 2 days after bombardment. This tissue was maintained for 2 weeks without being observed. During the 2 weeks the remaining tissue was examined under blue light on 6 separate occasions. At the end of this period, the number of GFP

positive cells in the rarely observed sample was similar to the number of GFP positive cells in the frequently observed sample. Repeated observation probably did not have a major detrimental effect. However, it is possible that the first observation was uniquely destructive and responsible for the sharp decline in GFP positive cells between 2 and 3 days after bombardment.

The GFP positive cells were not randomly dispersed across the tissue but were clumped in small areas of up to fifty expressing cells (expression clumps). The GFP positive cells, even within expression clumps, were rarely contiguous but were typically isolated cells. Despite the rapid growth of the cell culture over the period of observation, there was no evidence that the GFP positive cells were multiplying because they remained isolated, single cells. Eady et al. (2) have described a broad meristematic zone in the upper cell layers of similar cell cultures, suggesting that replication should have been observed in some GFP positive cells. However, it is possible that some surface cells are not part of this meristematic zone.

Lack of division might also be due to damage caused by the bombardment. To test whether the bombardment was severely destructive, bombarded and unbombarded cell cultures were stained with propidium iodide (propidium iodide (PI) accumulates in the nucleus of damaged and dead cells). There was no obvious difference in PI staining between bombarded and unbombarded tissue. However, both tissues had many cells on the surface that took up the PI stain, suggesting that those cells that received DNA by bombardment were already in the process of dying. Attempts to correlate transient expression with PI uptake failed (the assay for β-glucuronidase activity killed all cells and GFP activity made it impossible to view PI uptake).

2. 3. 2. *Ds* excision in onion cells mediated by transient transposase expression.

To test if transient transposase expression could mediate *Ds* excision in onion cells, *pSLJ1101* and *pNT103* were co-bombarded into onion cell cultures. *Ds* excision from *pNT103* would have resulted in expression of the *uid*A gene carried on *pNT103*. As a negative control, tissue was co-bombarded with *pNT103* and *pSLJ[EcoRI]* (a deletion derivative of *pSLJ1101* lacking the full transposase gene). As a positive control, tissue was

co-bombarded with *pNT[BamHI]* (a deletion derivative of *pNT103* lacking the *Ds* element) and *pSLJ1101*. Three petri dishes with a 200mm^2 area of tissue were individually bombarded per treatment. After 5 days all the tissue was assayed for β-glucuronidase activity. An average of 3 localised areas of β-glucuronidase activity were detected per petri dish in both the test and negative control treatments. The positive control treatment had 282 localised areas of β-glucuronidase activity. This experiment was repeated with similar results, suggesting that transient transposase expression from *pSLJ1101* was not mediating *Ds* transposition in this system.

A previous report suggested that *Ds* transposition was not as frequent after expression of the full length *Ac* transposase gene carried on *pSLJ1101* as after expression of a truncated transposase construct lacking the first two transposase ORFa translation start codons (10). A truncated transposase construct (*pNT804*) was obtained. One petri dish with a 200mm^2 area of tissue was bombarded per treatment (test = *pNT103* + *pNT804* and negative control = *pNT103*). After 5 days, the test and negative control tissue had, respectively, 48 and 2 localised areas of β-glucuronidase activity. In an expanded experiment, onion tissue on 8 petri dishes was bombarded with *pNT804* and *pNT103* and onion tissue on another 8 petri dishes was bombarded with *pNT103*. The average number of areas of localised β-glucuronidase activity for test and negative control treatments, respectively, was 10.9 and 1.6 per petri dish. These sample means were significantly different (ANOVA: $P<0.01$), suggesting that *pNT804* facilitates β-glucuronidase expression from *pNT103,* probably through transposase-mediated *Ds* transposition. To check that β-glucuronidase activity was not increased non-specifically by the addition of extra plasmid DNA, the experiment was repeated using co-bombardment of *pNT103* and *pSLJ[EcoRI]* as the negative control treatment. Again there was a significantly (approximately 6 fold) higher frequency of β-glucuronidase activity in the test treatment over the control treatment. It is likely that the specific function on *pNT804* that led to the enhanced frequency of *uid*A expression was the truncated *Ac* transposase gene. No further experiments were carried out to investigate *Ds* transposition in onion.

2. 4. Discussion.

Onion is recalcitrant to stable transformation. Despite this recalcitrance, transient transgene expression in onion is easily obtainable by both direct and *A. tumefaciens*-mediated gene delivery to a variety of onion tissue and cell cultures (16). Why is this expression only transient? It seems likely that the vast majority of transiently expressing cells simply died. The cultured cells transiently expressing GFP were not actively dividing. It is possible that these cells were damaged by the transformation process as has been suggested in another bombardment system (12). Alternatively, as is suggested by propidium iodide staining, many of the surface cells are naturally in the process of dying. The extent to which other factors, such as DNA loss and gene silencing, are responsible for the transient nature of gene expression in onion cells remains unexplored. If the results from my experiments on transient T-DNA expression in *N. plumbaginifolia* (Chapter 4) are generally applicable to other plants, then cell death, gene silencing and total loss of transferred DNA are all responsible for the loss of expression. Given the success that was achieved by tracking individual tobacco cells, it could be worthwhile using such a system to examine transient expression in onion.

A preliminary investigation of *Ds* transposition in onion cells was undertaken. Co-bombardment of *pSLJ1101* and *pNT103* produced no evidence of *Ds* transposition. However, co-bombardment of *pNT804* and *pNT103* produced a statistically significant, and repeatable, 6-fold increase in the frequency of β-glucuronidase activity. These results suggest that transient transposase expression can mediate *Ds* excision in onion cells. While neither *pNT804* alone, nor *pNT103* (alone or with *pSLJ[EcoRI]*) caused a high frequency of *uid*A expression, it is possible (but extremely unlikely) that some function of *pNT804*, other than the transposase gene, acted in conjunction with *pNT103* to enhance *uid*A expression.

The results also suggest that, in onion cells, *Ds* transposition is more frequent after expression of the truncated transposase gene than after expression of the full-length transposase gene. However, the length of the transposase gene is not the only difference between *pSLJ1101* and *pNT804* (eg. they have different promoters driving the transposase gene). The plasmid *pNT806* (the same as *pNT804* but with the full length *Ac* transposase untranslated leader sequence) was obtained to test the assumption that the truncated

transposase gene was more effective than the full length gene and as a potentially more useful negative control vector than *pSLJ[EcoRI].* However, the experiment has yet to be repeated using this vector. Neither the assumption that *uid*A expression resulted from *Ds* excision nor the assumption that transposition occurred while *pNT103* was extrachromosomal, can be confirmed without recovering the excision site or the *Ds* element.

The observation that transfer of *pNT804* (carrying the truncated transposase gene) was more effective at producing β-glucuronidase activity than *pSLJ1101* (carrying the full-length transposase) is in agreement with previous reports of transposition in tobacco (10). In tobacco, transfer of the truncated transposase gene resulted in a higher frequency of *Ds* excision from extrachromosomal plasmid DNA than transfer of the full-length gene. However, Shen and Hohn (17) observed the opposite result when they examined *Ds1* transposition from a replicating (maize streak virus) extrachromosomal vector in maize plants. They found a higher frequency of transposition when the transposase was encoded by the full-length gene. They suggest several possible reasons for these contradictory results. Firstly, transposition from a non-replicating vector may be fundamentally different from transposition from a replicating vector. Secondly, there may be differences in the *Ds* elements (*Ds1* as opposed to simple *Ds* elements). Finally, the contradictory results may be due to a difference between transposition in dicots and in maize (a monocot). I examined transposition of a simple *Ds* element from a non-replicating vector in the monocot onion. If the enhanced *uid*A expression I observed in onion was due to employing a truncated rather than a full-length transposase gene, then that effect is not specific to dicots.

Wirtz et al. (20) examined *Ds* transposition from extrachromosomal DNA in maize. Interestingly, they found that *Ds* transposition was absolutely dependent on replication of the *Ds* vector. Their results are directly contradictory to previous observations of *Ds* excision from vectors lacking known replication functions (10, 11, 13, 15, 18). The results presented here suggest that, in onion, *Ds* transposition from plasmid DNA does not require the presence of plant replication functions on the plasmid DNA. Shen and Hohn (17) and Wirtz et al. (20) examined *Ds* transposition in maize. Both groups obtained results that contradicted other studies of *Ds* transposition in plants that are not natural hosts to *Ac/Ds*

elements. It is possible that *Ds* transposition in maize is qualitatively different from *Ds* transposition in unnatural plant hosts (17).

Development of a *Ds*-mediated transformation system for onion was terminated primarily because, at the time the decision was made, no measurable frequency of stable expression could be demonstrated following direct gene transfer to cultured onion cells. The frequency of stable expression following co-cultivation was rare and erratic. Also, there was no certainty that *Ds* transposition would increase transgene integration. Further, given my observation that transiently expressing onion cells were not dividing, it seemed unlikely that the lack of stable expression was entirely due to a lack of transgene integration. A high probability existed that applying the *Ds* integration system would not have led to the recovery of transformed tissue. Without being able to recover and examine stably transformed tissue it seemed probable that no further conclusions would be attainable. We further decided that understanding the underlying causes of loss of transgene expression was more important than applying the *Ds*-mediated integration system to onion.

Since that time, a considerable amount of research has produced a reliable *A. tumefaciens*-mediated onion transformation system (6). There is also now experimental evidence that, in a significant minority of cases, lack of stable T-DNA integration is responsible for lack of recovery of stably transformed plants in some plant systems (Chapter 3 and 4). Testing a *Ds*-mediated transformation system in onion, where the *Ds* element is carried on T-DNA, is certainly now feasible and worthwhile given the measurable but low ratio of stable to transient transgene expression currently achievable. The advances in onion transformation that have recently occurred suggest it is likely that a measurable frequency of stable transformation could be obtained using biolistic delivery. If so, the question of whether or not *Ds* transposition (following direct DNA transfer) leads to higher stable transformation frequencies in onion may now be answerable.

In order to test the theory that *Ds* transposition from extrachromosomal plasmid DNA to chromosomal DNA could increase the stable transformation frequency of onion, a suitable vector (*pRJW28*) was constructed. *pRJW28* contains the simple *Ds* element from *pMDSBAR* (15) inserted between the CaMV35S promoter and the *uid*A gene of *pART8*.

Thus it should be possible to visualise *Ds* excision from *pRJW28* by staining for β-glucuronidase activity. The expression cassette from *pART7* (CaMV35S promoter-multiple cloning site-ocs transcription termination sequence) was inserted within the *Ds* element to allow the inclusion of a reporter gene (such as *m-gfp5-ER*) or a selectable gene (such as *bar*). The structure of this construct was confirmed by restriction mapping. This vector could be used in any further efforts to develop a *Ds*-mediated integration system in onion.

2. 5. References

1. Eady C: Towards the transformation of onions (*Allium cepa*). New Zealand Journal of Crop and Horticultural Science 23: 239-250 (1995).

2. Eady CC, Butler RC, Suo Y: Somatic embryogenesis and plant regeneration from immature embryo cultures of onion (*Allium cepa* L.). Plant Cell Reports 18: 111-116 (1998).

3. Eady CC, Lindsey K, Twell D: Differential activation and conserved vegetative cell-specific activity of a late pollen promoter in species with bicellular and tricellular pollen. The Plant Journal 5: 543-550 (1994).

4. Eady CC, Lister CE: A comparison of four selective agents for use with *Allium cepa* L. immature embryos and immature embryo-derived cultures. Plant Cell Reports 18: 117-121 (1998).

5. Eady CC, Lister CE, Suo Y, Schaper D: Transient expression of *uid*A constructs in *in vitro* Onion (*Allium cepa* L.) cultures following particle bombardment and *Agrobacterium*-mediated DNA delivery. Plant Cell Reports 15: 958-962 (1996).

6. Eady CC, Weld RJ, Lister CE: *Agrobacterium tumefaciens*-mediated transformation and regeneration of onion (*Allium cepa* L.) plants. Plant Cell Reports (in press).

7. Gleave AP: A versatile binary vector system with a T-DNA organisational structure conducive to efficient integration of cloned DNA into the plant genome. Plant Molecular Biology 20: 1203-1207 (1992).

8. Goldsbrough AP, Tong Y, Yoder JI: *Lc* as a non-destructive visual reporter and transposition excision marker gene for tomato. The Plant Journal 9: 927-933 (1996).

9. Haseloff J, Siemering KR, Prasher DC, Hodge S: Removal of a cryptic intron and subcellular localization of green fluorescent protein are required to mark transgenic *Arabidopsis* plants brightly. Proceedings of the National Academy of Science USA 94: 2122-2127 (1997).

10. Houba-Herin N, Domin M, Leprince A-S: Some features about transposition of the maize element *Dissociation* in *Nicotiana plumbaginifolia*. Genetica 93: 41-48 (1994).

11. Houba-Herin N, Domin M, Pedron J: Transposition of a *Ds* element from a plasmid into the plant genome in *Nicotiana plumbaginifolia* protoplast-derived cells. The Plant Journal 6: 55-66 (1994).

12. Hunold R, Bronner R, Hahne G: Early events in microprojectile bombardment: cell viability and particle location. The Plant Journal 5: 593-604 (1994).

13. Kunze R, Kuhn S, Jones JDG, Scofield SR: Somatic and germinal activities of maize *Activator* (*Ac*) transposase mutants in transgenic tobacco. The Plant Journal 8: 45-54 (1995).

14. Laufs J, Wirtz U, Kammann M, Matzeit V, Schaefer S, Schell J, Czernilofsky AP, Baker B, Gronenborn B: Wheat dwarf virus *Ac/Ds* vectors: expression and excision of transposable elements introduced into various cereals by a viral replicon. Proceedings of the National Academy of Science USA 87: 7752-7756 (1990).

15. Lebel EG, Masson J, Bogucki A, Paszkowski J: Transposable elements as plant transformation vectors for long stretches of foreign DNA. Theoretical and Applied Genetics 91: 899-906 (1995).

16. Sambrook J, Fritsch EF, Maniatis T: Molecular cloning. A laboratory manual. Ed. 2. Cold Spring Harbour Laboratory Press, (1989).

17. Shen WH, Ramos C, Hohn B: Excision of *Ds1* from the genome of maize streak virus in response to different transposase-encoding genes. Plant Molecular Biology 36: 387-392 (1998).

18. Shimamoto K, Miyazaki C, Hashimoto H, Izawa T, Itoh K, Terada R, Inagaki Y, Iida S: *Trans*-activation and stable integration of the maize transposable element *Ds* cotransfected with the *Ac* transposase gene in transgenic rice plants. Molecular and General Genetics 239: 354-360 (1993).

19. Sugimoto K, Otsuki Y, Saji S, Hirochika H: Transposition of the maize *Ds* element from a viral vector to the rice genome. The Plant Journal 5: 863-871 (1994).

20. Wirtz U, Osborne B, Baker B: *Ds* excision from extrachromosomal geminivirus vector DNA is coupled to vector DNA replication in maize. The Plant Journal 11(1): 125-135 (1997).

Chapter 3.

Ds transposition Mediated by Transient Transposase Expression in *Hieracium aurantiacum.*

3. 1. Introduction

Ac/Ds transposons from *Zea mays* have been used in attempts to isolate genes by insertional mutagenesis in a wide variety of heterologous plants (1, 2, 8, 13, 22, 23, 41, 53). Typically the *Ds* transposon is used as the transposable element while the *Ac* transposase gene is encoded on a separate construct. The elements of this binary system are transferred into separate cells from which plants are regenerated. The elements can then be brought together to allow *Ds* transposition by crossing the two transformed plant lines. Subsequently, the *Ds* transposon can be restabilised by segregating the two unlinked elements. By separating transformation and regeneration from selection for *Ds* transposition, researchers avoid confusing mutations caused by *Ds* insertion with mutations caused by tissue culture and transformation (somaclonal variation) (2).

In this laboratory, heterologous transposon tagging is being developed for gene isolation in the small herbaceous plant *H. aurantiacum*. *H. aurantiacum* is being used as a model system to study the genetics of apomixis. To this end, work has previously been carried out to optimise *H. aurantiacum* micropropagation and transformation (4, 5). As part of the investigation into gene tagging in *H. aurantiacum*, the practicality of using transient transposase expression to mobilise a chromosomally integrated *Ds* element without stable integration of the transposase construct has been explored. In this gene tagging strategy, T-DNA encoding the *Ac* transposase gene is transferred to plant cells. The plant cells have a chromosomal locus with a *Ds* element inserted between a plant promoter and a spectinomycin resistance gene. When transient transposase expression induces *Ds* transposition, the spectinomycin resistance gene will be expressed. Cells in which the *Ds* element transposed can be selectively propagated by culture on a medium containing spectinomycin (Figure 1.1.2).

Transient transposase expression is sufficient to mobilise extra-chromosomal *Ds* elements (eg. 3, 19, 21, 27, 31, 40) and chromosomally-inserted *Ds* elements (eg., 12). By using

transient transposase expression to mobilise a chromosomally-inserted *Ds* element it may be possible to achieve stabilisation of the reintegrated *Ds* element shortly after transposition through loss of transposase expression. Other potential advantages of this gene tagging strategy are gene tagging in an isogenic background, and rapid generation of independent transposition events without the requirement of a sexually mature plant. Unlike the co-transformation gene tagging strategy, where the transposase source and the transposon are simultaneously transferred to the same plant cell (14, 20, 45), this new strategy should allow the *Ds* excision locus to be genetically linked to the target gene prior to gene tagging. Linking the *Ds* locus to the target gene increases the probability of the *Ds* element inserting into the target gene. This strategy does, however, retain some of the problems (mutations caused by transformation and somaclonal variation) associated with gene tagging through co-transformation.

As well as developing a novel gene tagging strategy, this work had two further objectives. Firstly, *H. aurantiacum* provided an ideal model plant system to test and develop the components of the *Ds*-mediated transgene integration system that was to be applied to onion transformation. Secondly, I wanted to test the assumption that transient T-DNA expression was due to transcription from extrachromosomal T-DNA molecules that were subsequently lost from the cell. If that hypothesis was true, then in a sample of cells that had transiently expressed a T-DNA, it should be possible to find cells that lacked the T-DNA. *Ds* transposition after transient expression of the *Ac* transposase gene carried on T-DNA provided a method for enrichment of cells that had transiently expressed the T-DNA.

An overview of the strategy used to induce *Ds* transposition is provided (Figure 1. 1. 2 and Figure 1. 1. 3). Particle bombardment and co-cultivation with *A. tumefaciens* were examined as methods to deliver the *Ac* transposase gene to *H. aurantiacum.* In these experiments the transferred DNA carried the *uid*A gene and the frequency of β-glucuronidase expression was measured to estimate the efficiency of DNA transfer. Co-cultivation with *A. tumefaciens* was selected as the method for delivering the *Ac* transposase gene. The *Ac* transposase gene was delivered to *H. aurantiacum* leaf tissue stably transformed with the promoter/*Ds*/spectinomycin-resistance-gene construct (as described in Figure 1. 1. 2). Inclusion of the conditional, dominant, lethal gene *cod*A or

the visible marker gene *uid*A on the T-DNA carrying the *Ac* transposase gene provided a rapid method to test regenerants for the presence of the *Ac* transposase T-DNA.

3. 2. Materials and Methods

3. 2. 1 Plasmids

pSLJ3621

pSLJ3621 is a binary Ti vector containing a *Ds* element inserted into the untranslated leader sequence of the *aad*A gene (encoding resistance to spectinomycin) (Figure 3. 3. 1). The *Ds* element prevents transcription of the *aad*A gene. This plasmid was constructed by Bernie Carroll at the Sainsbury Laboratory, Norwich, UK.

pNE5 and *pSLJ1111*

pNE5 (45) and *pSLJ1111* (39) are binary vectors carrying the *Ac* transposase gene transcribed from the 35S promoter. The conditional lethal dominant gene *cod*A encoding cytosine deaminase is encoded on *pNE5* T-DNA to allow elimination of plants that stably integrate *pNE5* T-DNA. *uid*A (encoding β-glucuronidase) is encoded on the T-DNA of *pSLJ1111* as a marker gene.

pNT804

pNT804 (21) contains an *Ac* transposase gene construct consisting of the *Ac* transposase gene downstream of the 2' promoter of the octopine T-DNA. This truncated transposase fragment lacks ORFa ATG1 and ATG2 translation start sites.

pART8

pART8 is *pART7* (16) with the *uid*A gene (encoding β-glucuronidase) inserted at the multi-cloning site (*Xho*I-*Bam*HI) downstream of the CaMV 35S promoter.

pSLJ721(EIK)

pSLJ721 (38) contains an *Ac* element separating a *uid*A gene from an upstream CaMV 35S promoter. *pSLJ721(EIK)* is *pSLJ721* cleaved at the *Eco*RI site, the 5' overhang removed by

end filling (klenow enzyme) and religated, creating a frameshift in the coding region of the *Ac* transposase gene to produce a non-autonomous *Ds* element.

3. 2. 2. Plant material.

Ross Bicknell at New Zealand Institute for Crop and Food Research (Lincoln) provided plant material used in this study. Two *H. aurantiacum* varieties (a triploid (A3) variety and a tetraploid (R4) variety) and transgenic plants (A3 3621 plants) regenerated from A3 tissue co-cultivated with *A. tumefaciens* (*pSLJ3621*) were used.

3. 2. 3. Cell growth and co-cultivation conditions.

Hieracium aurantiacum stably transformed with *pSLJ3621*(A3 3621 plants) leaf explant was sterilized in 25% bleach for 35 minutes. *A. tumefaciens* LBA4404 (5) containing the binary vector *pSLJ1111* or *pNE5* was incubated over-night in a shaking incubator at 28^0C in 50 ml Luria-Bertani (LB) medium (37) with 100ug/ml kanamycin to select for the binary vectors. A3 3621 leaf explant was immersed in 20 ml *A. tumefaciens* saturated culture for several minutes, blotted on filter paper to remove excess *A. tumefaciens* and transferred to shoot induction (HR) medium (4) for co-cultivation in the dark. After 3 days co-cultivation, leaf explant was transferred to HR medium supplemented with timentin (200mg/l) to eliminate bacterial cells and spectinomycin (600mg/l) to select cells containing *Ds* transposition events. All shoots that emerged under spectinomycin selection were removed and transferred to root induction (HO) medium (4) supplemented with spectinomycin (600mg/l) and timentin (200mg/l). Spectinomycin resistant plants that formed roots under spectinomycin selection were maintained on HO medium, without selection, with monthly sub-culture.

Leaf explants from greenhouse-grown and *in vitro H. aurantiacum* plants stably transformed with *pSLJ721(EIK)* (R4 721 plants) were co-cultivated with LBA4404 containing *pNE5* (as described above). After co-cultivation, the tissue was maintained on HR medium supplemented with 200mg/l timentin and 600mg/l spectinomycin. To test for

Ds excision the tissue was sampled periodically and histochemically analysed for β-glucuronidase activity (as described below)

3. 2. 4. Bombardment conditions

All bombardments were with a Biolistic PDS-1000/He particle delivery system (Biorad). Very young leaves were removed from greenhouse grown A3 3621 plants and precultured on HR medium for 3 days prior to bombardment. Plasmolysis was induced 5 hours prior to bombardment and maintained for 16 hours after bombardment by culture on HR medium supplemented with 0.4M mannitol. Plasmid DNA was adsorbed to gold particles (mean diameter of 1μm) by $CaCl_2$ (0.96M) and spermidine (0.015M) precipitation. The tissue was bombarded in a vacuum (25 inches of mercury) at a target distance (stop grid to leaf explant) of 8cm, and a rupture disk pressure of 1550-1800 psi.

3. 2. 5. DNA extraction for PCR and Southern Blot analysis.

The midrib was removed from 1-3 young leaves and discarded. The remaining leaf tissue was frozen in liquid nitrogen and ground in 600μl urea extraction buffer (urea 42%, 0.3M NaOH, 0.1M Tris-HCl pH 8.0, 0.02M EDTA pH 8.0, Sarcosine 20%). DNA was purified by standard phenol/chloroform extraction, isopropanol precipitation, and desalted in 70% ethanol (38). RNA was digested in RNaseA for 60 minutes at 60^0C.

3. 2. 6. Southern blot analyses.

DNA (10ug) was digested with the appropriate restriction enzyme (Boehringer Mannheim) and size-fractionated by electrophoresis in 1% agarose gels in 1/2x Tris-borate-EDTA (1/2xTBE) buffer, transferred to nylon membrane (BioRad "Zeta probe" or Qiagen "Qiabrane") using capillary transfer (37) and hybridized to ^{32}P-dCTP- labelled probes at 65^0C for 12-24 hr. Prehybridisation and hybridisation was in a Techne oven in 15mls buffer (0.015g di-sodium dihydrogen pyrophosphate, 1.5g dextran sulphate, 1.5mls 10% SDS, 1.5mg denatured herringsperm DNA). The probe for transposase T-DNA was an 850bp fragment of *pSLJ1111* amplified by PCR with *Taq* polymerase (Boehringer Mannheim) using primers internal to the *Ac* transposase gene (5'-GTG ATA AGT CTT

GGG CTC TTG G -3' and 5'-TTG ATA TGC ACA AAA GAT TGG G-3'). The probe for the *Ds* element was a PCR fragment amplified with *Taq* polymerase (Boehringer Mannheim) using primers specific to the *bar* gene carried on the *Ds* element of *pSLJ3621*. The probe for the *Ds* locus was a PCR fragment amplified with *Taq* polymerase (Boehringer Mannheim) using primers specific to the *aad*A spectinomycin resistance gene (5'-TCC GCG CTG TAG AAG TCA CC-3' and 5'-GCT TCA AGT ATG ACG GGC TCA-3'). These PCR-generated probe fragments were purified by electrophoresis in a 0.7% agarose gel and extracted with the Qiaquick gel extraction kit (Qiagen). All probes were labelled by incorporation of ^{32}P-dCTP using a random priming kit (Amersham) Restriction digestion, ^{32}P-dCTP-labelling, PCR, and probe isolation were done according to the manufacturers' instructions. Final washing was performed in 1 X SSC, 0.1% SDS or 0.5 X SSC, 0.1% SDS at 65^{0}C. Hybridization patterns were visualized by phosphor-imaging on a Storm 840 laser scanner (Molecular Dynamics).

3. 2. 7. PCR analyses and sequencing

Polymerase chain reactions were normally performed on approximately 100ng total genomic DNA in a Techne Thermocycler PHC-3 with 0.625 units of *Taq* polymerase (Boehringer Mannheim) in a total volume of 25µl. Amplification of the *Ac* transposase gene (primers: 5'-GTG ATA AGT CTT GGG CTC TTG G -3' and 5'-TTG ATA TGC ACA AAA GAT TGG G-3') was for 35 cycles of 1 minute at 94^{0}C, 1 minute at 60^{0}C, 1 minute at 72^{0}C. The 35 amplification cycles were preceded by 1 cycle for 1 minute at 94^{0}C and followed by 1 cycle of 72^{0}C for 8 minutes. Amplification of the *Ds* excision site (primers: 5'-CGC GTT CAA AAG TCG CCT AAG GTC-3' and 5'-TCA CTG TGT GGC TTC AGG CCG CC-3') was the same as amplification of *Ac* transposase. PCR fragments amplified from the *Ds* excision site were purified by electrophoresis on 0.7% agarose gel and extracted with the 'Qiaquick' gel extraction kit (Qiagen). The sequencing was by 'Big Dye Cycle Sequencing' (Perkin Elmer) as per manufacturer's instructions and the sequencing reaction was run on a sequencing gel and the sequence information collected by the Waikato Sequencing Facility (Private bag 3105, Hamilton, New Zealand).

3. 2. 8. *uidA* and *codA* Expression.

uidA expression was monitored by histochemical localisation of β-glucuronidase activity. Plant tissue was incubated over night in 0.1M phosphate buffer containing: 1mM 5-bromo-4-chloro-3-indolyl β-D-glucuronide (x-glu); 2.5mM potassium ferricyanide and 2.5mM ferrocyanide (11). To test for *cod*A expression, shoots were cultured for several weeks on root induction medium supplemented with 500ug/ml 5-fluorocytosine (15).

3. 3. Results

3. 3. 1. Optimisation of transformation procedures.

For delivery of the *Ac* transposase gene to *H. aurantiacum*, particle bombardment was examined by quantifying β-glucuronidase activity following transfer of *pART8*. Several factors significantly improved the expression frequency (the number of cells with transient expression per cm^2 of leaf tissue) following particle bombardment. Prior to DNA delivery, greenhouse grown leaf explant was cultured on shoot regeneration (HR) medium (MS salts, B5 vitamins, 3% sucrose, 6-benzylaminopurine and 0.5 mg/L indole-3-butyric acid) for 0, 1 or 3 days. HR medium stimulates cell division and somatic organogenesis. The expression frequency 2-5 days after bombardment increased with longer pretreatment on HR medium (see Table 3. 3. 1). There was a significant interaction (ANOVA: $p<0.05$, df=2) between preculturing on HR medium and plasmolysis (initiatied 5 hours prior to bombardment and maintained until 16 hours after bombardment by culturing the tissue on HR medium supplemented with 0.4M mannitol). Plasmolysis improved the expression frequency if the tissue was precultured on HR medium for 3 days prior to bombardment but decreased the expression frequency when the tissue was precultured for 0 days (Table 3. 3. 2). The expression frequency also increased with decreasing leaf size (developmental age) (Table 3. 3. 1).

Several bombardment parameters were also examined for their effect on expression frequency. Expression frequency increased with shorter particle flight distance (the distance bombardment particles travelled after acceleration and before they hit the tissue) (Table 3. 3. 1). However, increasing the particle velocity by increasing the rupture disc pressure (900psi, 1100psi and 1550psi), did not significantly increase the expression

frequency suggesting that decreasing the microcarrier flight distance increased the expression frequency by increasing the density rather than the velocity of particles.

Table 3. 3. 1. Factors that increased transient expression in *H. aurantiacum* after particle bombardment.

Treatment	**Average[1]**	**Sample size[2]**	**P[3]**
Pre-culture[4] (expression measured 2 days after bombardment)			**
0 days	9.2	4	
1 day	10.5	4	
3 days	54	4	
Target distance[5]			**
12cm	0.39	9	
9cm	0.96	9	
6cm	12.86	10	
Leaf size[6]			**
2cm or more	0.64	9	
1.5-2cm	1.89	9	
less than 1cm	9.6	9	

1: Average = the average number of areas of β-glucuronidase activity per cm^2.
2: Sample size = number of individual bombardments analysed per treatment.
3: P = probability that the differences in averages are due to random variation. Calculated by ANOVA. ** $P<0.01$.
4: Pre-culture = leaf discs were cultured on HR medium for 0, 1 or 3 days prior to bombardment. The number of areas of β-glucuronidase activity were counted 2 days after bombardment.
5: Target distance = distance from the point of ejection of the gold particles from the gene gun to the leaf tissue.
6: Leaf size = maximum width of the leaf from which tissue to be bombarded was taken.

Table 3. 3. 2. Interaction between pre-culture and osmotic protection and their effect on the frequency of transgene expression after particle bombardment.

	3 days pre-culture[1]	**0 days pre-culture[1]**
Plasmolysis[2]	87.1[3]	4.8[3]
No plasmolysis[2]	21.6[3]	13.6[3]

Analysis of variance

SOURCE	**P**
Osmotic protection.....	0.09
Pre-culture.....	0.02
Interaction between osmotic protection and pre-culture.....	0.04

1: Number of days leaf tissue was cultured on HR medium prior to bombardment.

2: Plasmolysis was induced 5 hours prior to bombardment and maintained for 16 hours after bombardment by maintaining leaf tissue on HR medium supplemented with 0.4M mannitol.

3: Average number of areas of β-glucuronidase activity per cm^2 leaf tissue. Includes data from two independent replicates.

Table 3. 3. 3. Factors that increased transgene expression in *H. aurantiacum* after co-cultivation with *A. tumefaciens* (*pSLJ1111*).

Treatment	Average[1]	Sample size[2]	P[3]
Pre-culture (expression measured 2 days after co-cultivation)			*
Pre-culture[4]	10.2	13	
No pre-culture[4]	0.4	14	
Pre-culture (expression measured 10 days after co-cultivation)			-
Pre-culture[5]	17.7	19	
No pre-culture[5]	21.73	18	
Edge of leaf disc bruised			**
Bruising[6]	66.7	7	
No bruising[6]	10.2	13	
Timentin concentration			*
50mg/l timentin[7]	31	12	
200-800mg/l timentin[7]	51	38	

1: Average = the average number of areas of β-glucuronidase activity per 8mm leaf disc.
2: Sample size = number of 8mm leaf discs analysed per treatment.
3: P = probability that the differences in averages are due to random variation. Calculated by ANOVA. ** $p<0.01$, * $p<0.05$, - $p>0.05$.
4: Pre-culture = leaf discs were cultured on HR medium for 3 days prior to co-cultivation with *A. tumefaciens*. No pre-culture = leaf discs were not cultured on HR medium prior to co-cultivation with *A. tumefaciens*. The number of areas of β-glucuronidase activity were counted 2 days after co-cultivation.
5: Pre-culture = leaf discs were cultured on HR medium for 3 days prior to co-cultivation with *A. tumefaciens*. No pre-culture = leaf discs were not cultured on HR medium prior to co-cultivation with *A. tumefaciens*. The number of areas of β-glucuronidase activity were counted 10 days after co-cultivation.
6: Bruising = prior to cocultivation with *A. tumefaciens* leaf discs were damaged by squashing tissue inside the edge of the disc with an 8mm hole punch.
7: The concentration of timentin in the medium on which the leaf tissue was cultured after cocultivation with *A. tumefaciens*.

Co-cultivation with *A. tumefaciens* was also examined as a method for DNA delivery to *H. aurantiacum*. The expression frequency 2-5 days after co-cultivation with *A. tumefaciens* increased with pre-treatment on HR medium (see Table 3. 3. 3). However, pre-culturing plant tissue on HR medium prior to co-cultivation did not increase the expression frequency observed 10 days after co-cultivation, suggesting that pre-culturing decreased the time between co-cultivation and maximum *uid*A expression but did not increase the number of cells that eventually expressed *uid*A.

The effect of post-transformation exposure to spectinomycin (used to select for *Ds* excision events) and timentin (used to kill *A. tumefaciens* cells) on expression frequency was examined. Two weeks after co-cultivation with *A. tumefaciens*, expression frequency was not detectably changed by spectinomycin selection (600mg/l) and a broad range of concentrations of timentin (200-800mg/l) applied 3 days after DNA transfer (Table 3. 3. 3). However, reducing the timentin concentration to 50mg/l significantly decreased the expression frequency due to plant cell death probably caused by uncontrolled bacterial growth. Additional wounds created around the cut edge of the leaf discs by bruising the tissue with an 8mm diameter hole punch significantly increased expression frequency (Table 3. 3. 3).

The optimised bombardment protocol (see materials and methods) could produce expression frequencies of over 100 expressing cells per cm^2 of *H. aurantiacum* leaf tissue. However, 3 fold higher expression frequencies were regularly achieved by co-cultivation with *A. tumefaciens*. A further advantage of co-cultivation with *A. tumefaciens* was concentration of transient expression at the cut edge of the leaf tissue where shoot initiation generally occured. After bombardment, β-glucuronidase activity was distributed across the leaf surface. Frequently β-glucuronidase activity was predominantly observed in stomatal guard cells. Such differences in the distribution of transient expression events could greatly alter the proportion of transient expressers that could be recovered.

3. 3. 2. Transient *uid*A expression.

The ratio of cells that transiently expressed *uid*A to the number of cells that stably expressed *uid*A was quantified. *H. aurantiacum* leaf discs were co-cultivated with *A. tumefaciens* containing *pSLJ1111* (*uid*A). Samples of discs were stained for β-glucuronidase activity 7 and 26 days after co-cultivation. In one experiment, the number of areas of β-glucuronidase activity per disc declined from an average of 153 at 7 days to an average of 48 at 26 days. This decline was statistically significant (ANOVA, $p< 0.01$). In two other experiments no decline in the number of areas of β-glucuronidase activity was detected. These results suggest, at most, a modest decline in the number of cells expressing the T-DNA marker gene over the four weeks immediately following T-DNA transfer. The

low ratio of transient to stable T-DNA expression was a potential problem for the use of transient *Ac* transposase expression in gene tagging in *H. aurantiacum*. Ideally, transposase expression would be lost after *Ds* transposition creating a stable *Ds* integration locus.

3. 3. 3. Particle bombardment.

No evidence was found of *Ds* excision following delivery of a transposase construct to *H. aurantiacum* by particle bombardment. Leaf discs from *H. aurantiacum* plants previously transformed with the *Ds* source, *pSLJ3621* (A3 3621 plants), were bombarded with gold particles coated with the *Ac* transposase source, *pNT804*. As a negative control A3 3621 tissue was either not bombarded or bombarded with uncoated gold or gold coated with plasmid DNA lacking the *Ac* transposase gene (*pART8*). Shoots with low level resistance to spectinomycin (could form shoots but not roots on medium containing 600mg/l spectinomycin) were recovered in one of three experiments. Southern analysis of the shoots recovered after bombardment with *pNT804* demonstrated that the *Ds* element had not transposed. DNA from the recovered shoots was digested with *Hpa*I and *Bgl*II, size fractionated, transferred to a nylon membrane and hybridised to a probe homologous to the *aad*A gene (Figure 3. 3. 2). Where *Ds* excision had occurred, 5kb DNA fragments should have been detected. However, only 10kb fragments were expected if the *Ds* element had not excised and additional bands that could not result from precise excision of the *Ds* element were observed. The Southern produced evidence of other intragenomic events discussed in section 3.3.10.

3. 3. 4. *Ds* excision within R4721 plants after co-cultivation.

Following transfer of the *Ac* transposase gene to *H. aurantiacum* by co-cultivation with *A. tumefaciens*, evidence of *Ds* excision was observed. *H. aurantiacum* plants containing the T-DNA from the *Ds* source *pSLJ721(EIK)* (R4 721 plants) were regenerated. In several experiments, leaf tissue from the regenerated R4 721 plants was co-cultivated with *A. tumefaciens* carrying the *Ac* transposase source *pNE5* and maintained on HR medium. *Ds* excision in R4 721 cells would restore expression of the *uid*A gene encoding β-glucuronidase. Only one area of β-glucuronidase activity was observed in more than 200 8mm leaf discs suggesting a low frequency of *Ds* transposition (Figure 3. 3. 3).

3. 3. 5. *Ds* excision within A3 3621 plants after co-cultivation

Co-cultivation of *H. aurantiacum* plants, previously transformed with the *Ds* source *pSLJ3621* (A3 3621 plants), with *A. tumefaciens* containing the *Ac* transposase gene source *pSLJ1111* or *pNE5*, resulted in recovery of fully spectinomycin resistant shoots. In five separate experiments, leaf tissue from *H. aurantiacum* A3 3621 plants was co-cultivated with *A. tumefaciens* containing either *pSLJ1111* or *pNE5*. The leaf tissue was then cultured on a regeneration medium containing 600mg/l spectinomycin (HRS) (Figure 3. 3. 4). The negative control for these experiments was A3 3621 leaf explant that was not co-cultivated or co-cultivated with *A. tumefaciens* lacking the binary vectors *pSLJ1111* and *pNE5*. Over the five experiments, 132 independent shoots initially appeared, and were capable of being propagated, on HRS medium (Figure 3. 3. 5). From these shoots, 84 fully spectinomycin resistant plants (capable of shoot and root formation under spectinomycin selection) were regenerated (Figure 3. 3. 6). The remaining 48 shoots either died on HRS medium or were not capable of root formation on medium supplemented with spectinomycin (Figure 3. 3. 7).

In a selection of 14 of the 84 regenerated plants, the *pSLJ3621* T-DNA locus was amplified by PCR to confirm transposition of the *Ds* element. A PCR amplification product of the predicted size was detected from all fully spectinomycin resistant plants tested. In three plants the *Ds* excision site was sequenced to confirm that repair of the spectinomycin resistance gene was specifically due to *Ds* transposition rather than some other recombination event (Figure 3. 3. 8). The three excision sites had unique sequences indicating that the three plants had regenerated from independent excision events as expected. One plant contained a typical *Ds* excision footprint: the original 8bp target sequence duplication created by the *Ds* integration was retained with the two central bases being replaced by their complements. *Ds* excision in a second plant restored the original unduplicated target sequence. Target sequence restoration is also typical of *Ac*/*Ds* transposition (39). The excision site in the third plant contained a 54bp deletion extending in one direction from the unduplicated target sequence. Although such large deletions have only rarely been observed in association with *Ac*/*Ds* excision (39), the fact that the deletion extended precisely from the *Ds* integration site suggests the deletion resulted from transposition.

3. 3. 6. Transposase integration

In order to facilitate the selection of those regenerated plants in which the *Ds* element had transposed but did not retain transposase sequences, a marker gene (*codA* or *uidA*) was incorporated into the transposase T-DNA. Histochemical analysis for β-glucuronidase activity revealed that following co-cultivation with *A. tumefaciens* (*pSLJ1111*) and selection for spectinomycin resistance, 24.6% (17 out of 69) of spectinomycin resistant plants lacked β-glucuronidase activity (Figure 3. 3. 9). Of the plants that had visible β-glucuronidase activity, many had tissue (even whole leaves) with no visible activity. This result suggests that either the *uid*A gene was lost from some cells during plant regeneration, or the plants were derived from a mixture of transformed and untransformed cells, or the *uid*A gene was present but not expressed in some cells. Following co-cultivation with *A. tumefaciens* (*pNE5*), 26% (4 out of 15) of spectinomycin resistant plants lacked cytosine deaminase activity (Figure 3. 3. 10). Southern blotting and hybridisation to a P^{32}-dCTP-labelled probe homologous to the *Ac* transposase sequence was used to test for the presence of *pNE5* or *pSLJ1111* T-DNA. Total genomic DNA was digested with the restriction enzyme *Hin*dIII that was predicted to release a 1.6kb fragment from within the *Ac* transposase gene. Surprisingly, only 33% (7 out of 21) of spectinomycin resistant plants that did not express *uidA* or *codA* also lacked the 1.6kb *Hin*dIII fragment (eg. Figure 3. 3. 11, lanes 8, 9, 11-14). Thus, most plants that did not express the marker gene did retain the transposase gene.

3. 3. 7. *Ds* transposition

Ds transposition was detected by Southern hybridisation in 6 of the 7 plants in which the *Ac* transposase gene was not detected. Total genomic DNA from the 7 plants lacking transposase sequences and two plants that retained transposase sequences was digested with *Hpa*I and *Bgl*II and size fractionated by electrophoresis on a 1% agarose gel. After Southern transfer, the DNA was hybridised to a labelled probe homologous to the *aad*A gene. This probe would detect a 10kb fragment if the *Ds* element had not transposed and a 5kb fragment if transposition had occurred (Figure 3. 3. 1). 6 of the 7 plants had the predicted 5kb fragment (Figure 3. 3. 12, lanes 2, 3, 5-8). As the *Ac* transposase gene was not detected in these 6 plants (detectable at less than one copy per genome (Figure 3. 3. 11)), and *Ds* excision depends on transposase activity, the transposase source was most

likely lost from these plants after transient transposase expression. One of the 7 plants (Figure 3. 3. 12, lane 4) did not have the 5kb excision fragment, suggesting that *Ds* excision did not occur, the excision locus was subsequently lost or that the *Ds* element reintegrated within the *Hpa*I-*Bgl*II fragment. The 10kb fragment was also present in all samples, suggesting that there was more than one *Ds* (*pSLJ3621*) locus. One plant (Figure 3. 3. 12, lane 5) had two additional fragments: one larger and one smaller than the 5kb band. These fragments are probably the result of recombination not associated with *Ds* excision or integration of truncated *pSLJ3621* T-DNA in the original A3 3621 parental plant.

3. 3. 8. *Ds* transposition and chimaeric plants

For gene tagging it is desirable that all cells in the regenerated plants have the transposed *Ds* element. That is, the entire plant should be derived from a single cell in which the *Ds* element transposed. All A3 3621 shoots regenerated under spectinomycin selection after transfer of the *Ac* transposase gene source (*pSLJ1111* or *pNE5*) were maintained under selection on regeneration medium for several weeks, and in some cases for several months. No areas of bleaching were visible on leaves on HR medium, suggesting that the shoots were not chimaeric for *Ds* excision. Shoots that were maintained for several months on HO medium under spectinomycin selection did eventually bleach (Figure 3. 3. 6). However, this bleaching was not concentrated in sectors but covered most newly emerging leaves and was probably indicative of general, slow loss of chlorophyll due to loss of high *aad*A expression over time. Leaves that gradually bleached formed vigorous green shoots when transferred to HR medium supplemented with 600mg/l spectinomycin, demonstrating that this tissue still retained the *Ds* excision locus. Further, the signal intensity in the autoradiographs of *Bgl*II/*Hpa*I digested DNA suggested that the 10kb fragment and the 5kb fragment were present in roughly equal numbers. Equal signal intensity suggested that there were two *pSLJ3621* T-DNA loci. If the plants were chimeric for *Ds* transposition, then variation in signal intensity would be expected (Figure 3. 3. 12).

3. 3. 9. Control plants

Ds transposition did not occur in tissue not exposed to the *Ac* transposase gene. No fully spectinomycin resistant plants were recovered from control tissue that was not co-cultivated with *A. tumefaciens*. However, in one experiment, 4 spectinomycin resistant plants were recovered on one petri dish from control tissue co-cultivated with an *A. tumefaciens* strain supposed to be lacking the transposase construct. DNA from these control plants was digested with *Hpa*I and *Bgl*II and hybridised to a probe homologous to the *aad*A gene to test for *Ds* excision. The 5kb fragment predicted to result from *Ds* excision was detected in all four plants (Figure 3. 3. 12, lanes 6-9). Histochemical analysis revealed that one of the four plants expressed β-glucuronidase suggesting that the transposase source *pSLJ1111* had been transferred to that plant. Southern blotting and hybridisation to a transposase probe demonstrated that the β-glucuronidase-expressing plant also contained *Ac* transposase sequences (Figure 3. 3. 11, lane 10) and that the three plants not expressing β-glucuronidase lacked transposase sequences (eg. Figure 3. 3. 11, lanes 9 and 14). As all four plants arose from the same control treatment batch, no other fully spectinomycin resistant plants were recovered from any other control treatment, and transposase sequences and β-glucuronidase activity were detected in one plant, it is concluded that all four plants arose from a control treatment contaminated with *A. tumefaciens* containing *pSLJ1111*. For this reason, these plants have been included in the results and analysis as test rather than control plants.

3. 3. 10. Somatic variation in parent tissue

Plants regenerated from A3 3621 leaf tissue without exposure to the transposase gene and without selection for spectinomycin resistance, frequently had evidence of recombination at the *Ds* locus that was not associated with *Ds* transposition. During the period when the co-cultivation experiments were carried out, some untransformed tissue from each parent plant was collected and *in vitro* plants regenerated without selection. Seven *in vitro* plants were independently regenerated from one parent plant (A3 3621#6). A3 3621#6 was the A3 3621 plant from which all but one of the spectinomycin resistant plants lacking transposase sequences were derived. DNA from six of the seven A3 3621#6 clones was digested with *Hin*dIII, size fractionated and hybridised to a labelled probe homologous to the *aad*A gene. *Hin*dIII does not cut within the *pSLJ3621* T-DNA and should release a

~12kb (or bigger) fragment from each intact *pSLJ3621* T-DNA insert. Although these plants were ostensibly clones, they did not all contain the same *Hin*dIII-*aad*A hybridisation pattern (Figure 3. 3. 13). Several of the bands were smaller than 12kb, suggesting either that only part of the *pSLJ3621* T-DNA was integrated or that recombination has resulted in partial loss of the T-DNA.

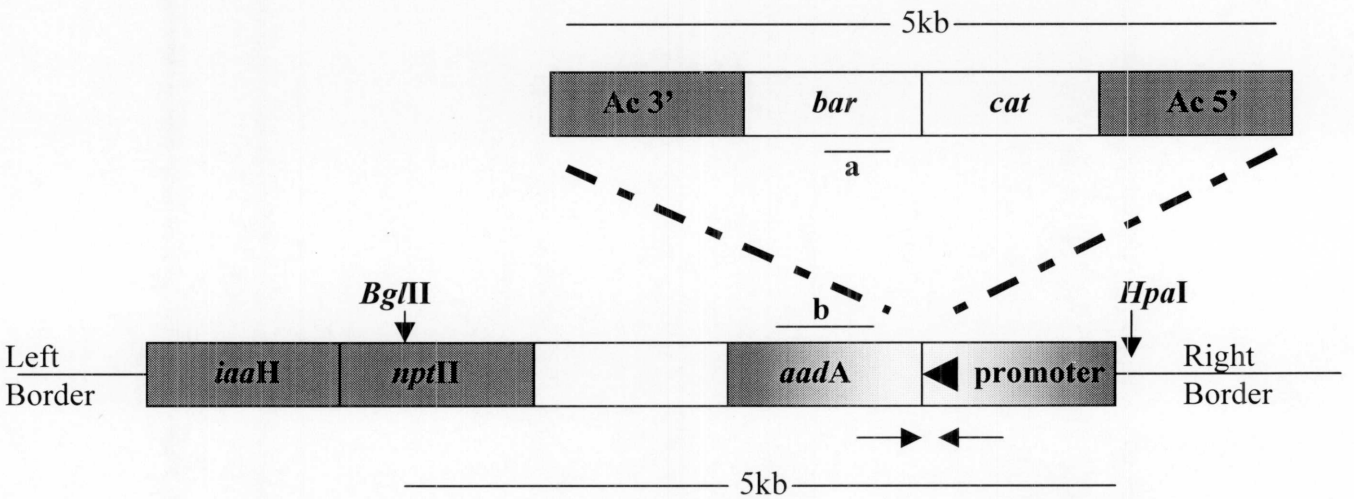
DNA from the seven A3 3621#6 clones was also digested with *Bgl*II and *Hpa*I, and hybridised to the *aad*A probe to test for *Ds* excision. Several different hybridisation patterns were again evident (Figure 3. 3. 14). None of the plants had the 5kb fragment predicted from *Ds* transposition. These results suggest that there has been a high frequency of recombination during the regeneration and maintainence of the *in vitro* A3 3621#6 plants. The recombination was not associated with precise excision of the *Ds* element in the plants examined.

3. 3. 11. *Ds* reintegration.

For gene tagging to work, the transposed *Ds* element must reintegrate into plant genomic DNA. The 6 plants lacking transposase sequences in which the *Ds* excised, and 14 plants retaining transposase sequences in which the *Ds* excised, were tested for *Ds* reintegration. DNA from the 20 plants was digested with *Hin*dIII, size fractionated by gel electrophoresis, transferred to a membrane and sequentially hybridised to probes homologous to the spectinomycin resistance gene (*aad*A) and the *bar* gene carried on the *Ds* element (Figure 3. 3. 15). If the *Ds* element transposed and reintegrated, the *Ds* probe should have hybridised to a DNA fragment that the *aad*A probe did not hybridise to and different bands should be visible on the two autoradiographs. This result was seen in 2 of the 6 plants lacking transposase sequences (Figure 3. 3. 15, lanes 12 and 13) and 9 of the 14 plants that retained transposase sequences (eg. Figure 3. 3. 15, lanes 5 and 7). This result suggests that *Ds* reintegration occurred from about half the transposition events which is consistent with previous reports of *Ds* reintegration in other plants (1, 8, 25, 43). However, in the negative control for this experiment (*Hin*dIII digested DNA from the A3 3621 plant) (Figure 3. 3. 15; lane 2), the *Ds* probe also hybridised to a band to which the *aad*A probe did not hybridise. This result is probably due to recombination unassociated with *Ds* transposition as the 5kb *Hpa*I/*Bgl*II DNA fragment resulting from *Ds* excision was

not detected in this A3 3621 clone (Figure 3. 3. 14, lane 1). Recombination unassociated with *Ds* transposition, rather than *Ds* reintegration after transposition, might account for separation of the *Ds* element from the spectinomycin resistance gene in some of the 11 plants described above (Figure 3. 3. 15). The results obtained were not sufficient to discriminate between these two possibilities. However, as it is expected that *Ds* excision did occur in the 11 plants, *Ds* reintegration is a more likely explanation. The *Ds* probe also hybridised to the A3 plant from which the A3 3621 plants were derived (Figure 3. 3. 15, lane1). This relatively weak, ~12kb band, unrelated to the *Ds* element, did not detract from the results. However, to confirm that the DNA fragments that hybridised to the *Ds* probe but not the *aad*A probe contained *Ds* sequences and not just genomic DNA, the same membrane (as in Figure 3. 3. 15) was re-hybridised to a new *Ds* probe (Figure 3. 3. 16). This probe hybridised to the *Ds* element but not to A3 genomic DNA and confirmed that the relevant fragments did contain *Ds* sequences.

Figure 3. 3. 1.
Map of T-DNA of plasmid *pSLJ3621*.



This map is not drawn to scale

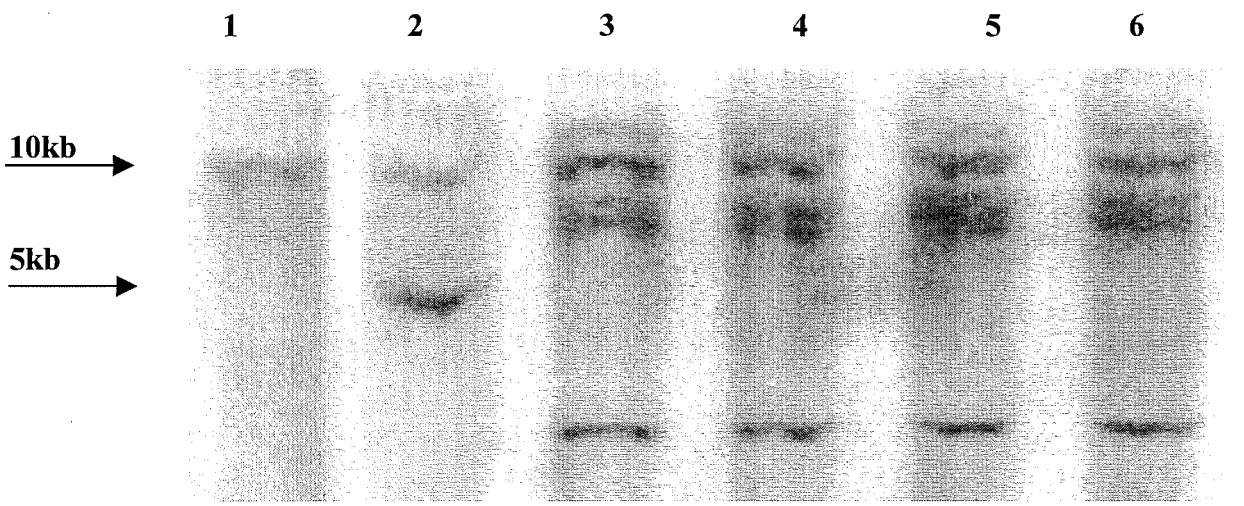
The *Ac* 3' and 5' terminal sequences define the *Ds* element that is inserted between the *aad*A spectinomycin resistance gene and its nos promoter.

a: probe for *bar* gene.

b: probe for spectinomycin resistance gene.

→ ← PCR primers for amplification of *Ds* excision site.

Figure 3. 3. 2.
Autoradiograph of *Hpa*I/*Bgl*II digested *H. aurantiacum* DNA hybridised to a labelled probe homologous to the *aad*A spectinomycin resistance gene.



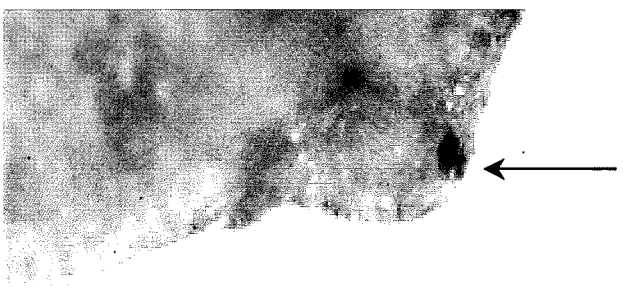
Lane 1: DNA from the parental plant A3 3621#6

Lane 2: DNA from a fully spectinomycin resistant plant derived from A3 3621#6 co-cultivated with *A. tumefaciens* (*pSLJ1111*) showing both the 10kb fragment and the 5kb fragment indicative of *Ds* transposition.

Lanes 3 and 4: DNA from partly spectinomycin resistant (form shoots but not roots on medium containing 600mg/l spectinomycin) plants derived from A3 3621 plants bombarded with *pNT804.*

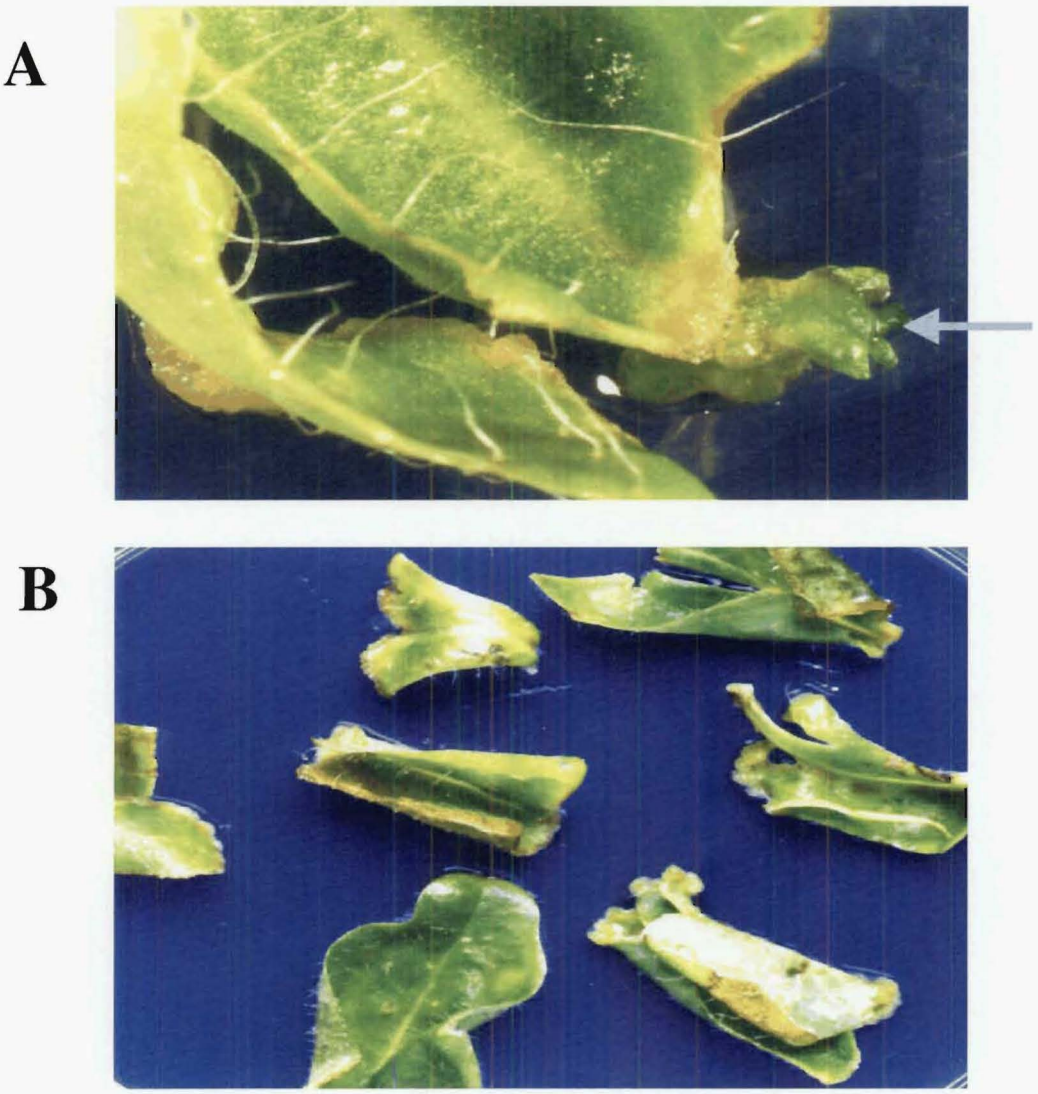
Lanes 5 and 6: DNA from partly spectinomycin resistant plants derived from A3 3621 plants co-cultivated with *A. tumefaciens* (*pSLJ1111*).

Figure 3. 3. 3.
H. aurantiacum R4 721 co-cultivated with *A. tumefaciensd (pNE5)* and stained for β-glucuronidase activity



Arrow shows blue spot resulting from β-glucuronidase activity that probably resulted from *Ds* transposition after transient expression from the *Ac* transposase gene encoded on *pNE5*.

Figure 3. 3. 4.
H. aurantiacum A3 3621 leaf explant cultured on HR medium, supplemented with 600mg/l spectinomycin, for four weeks.



A: Tissue co-cultivated with *A. tumefaciens* (*pSLJ1111*) with shoot regeneration (arrow) on HR medium supplemented with spectinomycin.

B: Negative control tissue showing no regeneration on HR medium supplemented with spectinomycin.

Figure 3. 3. 5.
H. aurantiacum A3 3621 shoots regenerating on HR medium supplemented with 600mg/l spectinomycin.

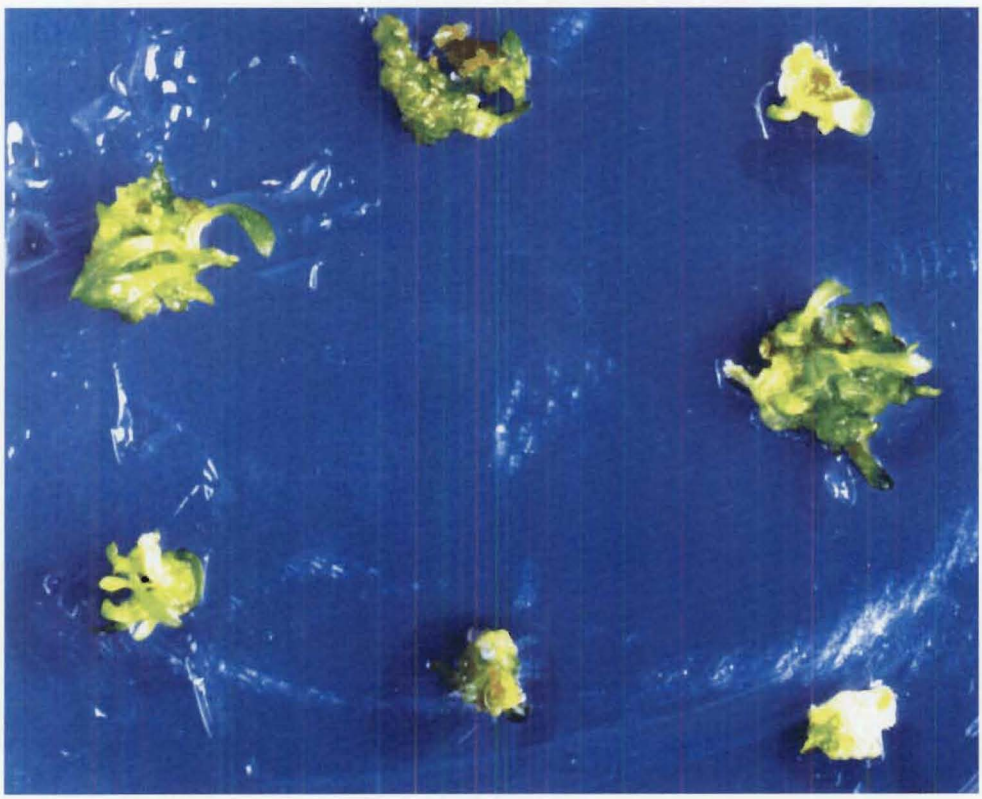
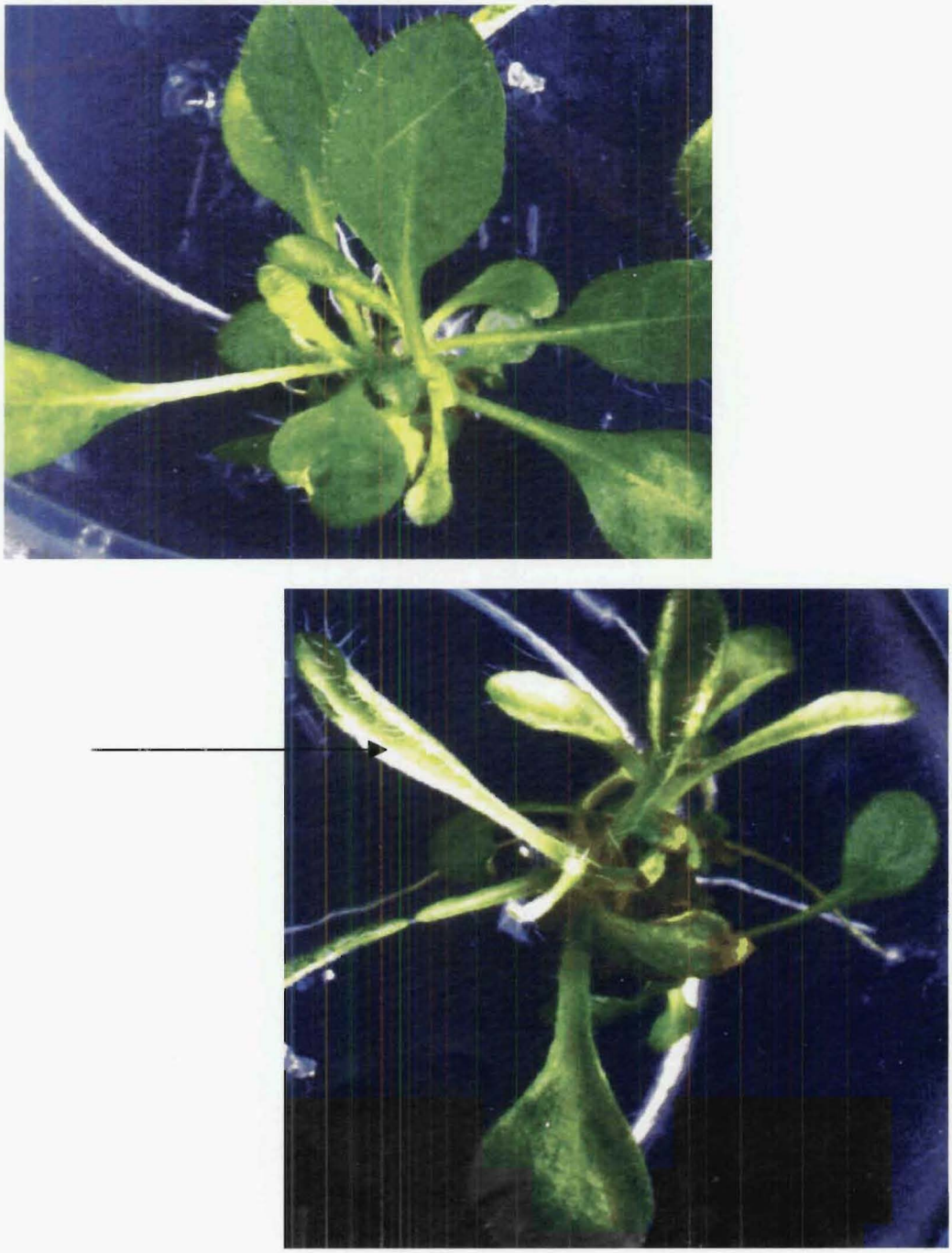


Figure 3. 3. 6.
H. aurantiacum A3 3621 shoots regenerated after co-cultivation with *A. tumefaciens* (*pSLJ1111*) and selection for spectinomycin resistance.

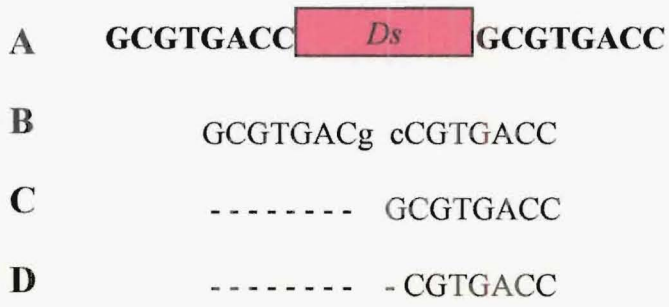


Some areas of bleaching are visible on leaves (arrow).

Figure 3. 3. 7.
H. aurantiacum A3 3621 shoots regenerated after co-cultivation with *A. tumefaciens* (*pSLJ1111*) not forming roots on HO medium supplemented with 600mg/l spectinomycin.

A3 3621 shoots regenerated on HR medium under spectinomycin selection. Stunted growth, bleaching and failure to produce roots when plated on HO medium supplemented with 600mg/l spectinomycin suggests these shoots are not fully spectinomycin resistant.

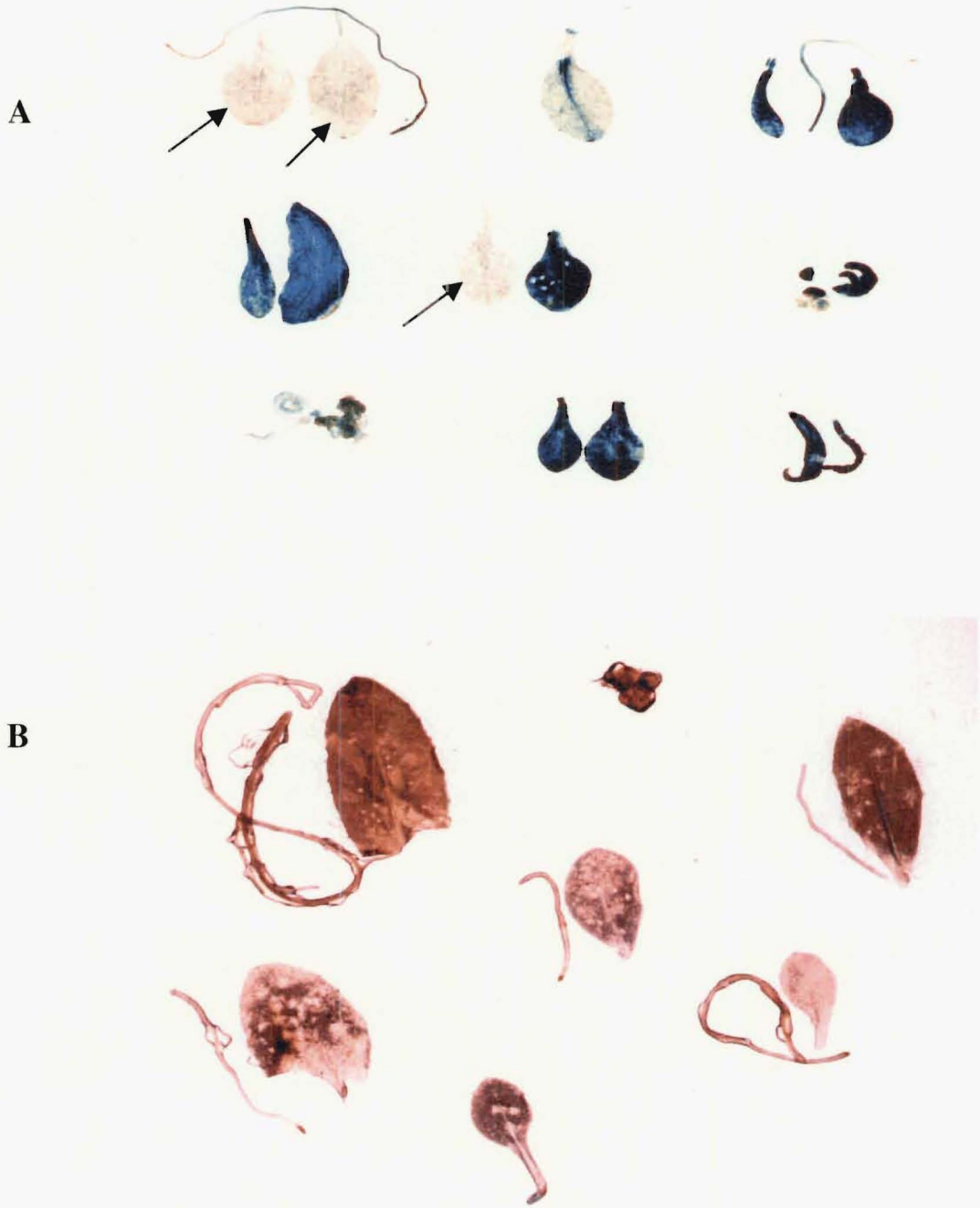
Figure 3. 3. 8.
DNA sequences of *Ds* excision sites in three spectinomycin resistant *H. aurantiacum* plants.



A: DNA sequence of the eight base pair repeat flanking the *Ds* element (represented by a red box) in the A3 3621 parental plants.

B, C and D: Sequences through the *Ds* excision site of three spectinomycin resistant plants. Lower case letters indicate base substitutions. Dash (-) indicates a deleted base. In C, the deletion precisely removed one of the eight base pair repeats. In D, the deletion removed one of the eight base pair repeats, one base pair from the other repeat and a further 42 base pairs.

Figure 3. 3. 9.
H. aurantiacum A3 3621 shoots regenerated after co-cultivation with *A. tumefaciens* (*pSLJ1111*), stained for β-glucuronidase activity.

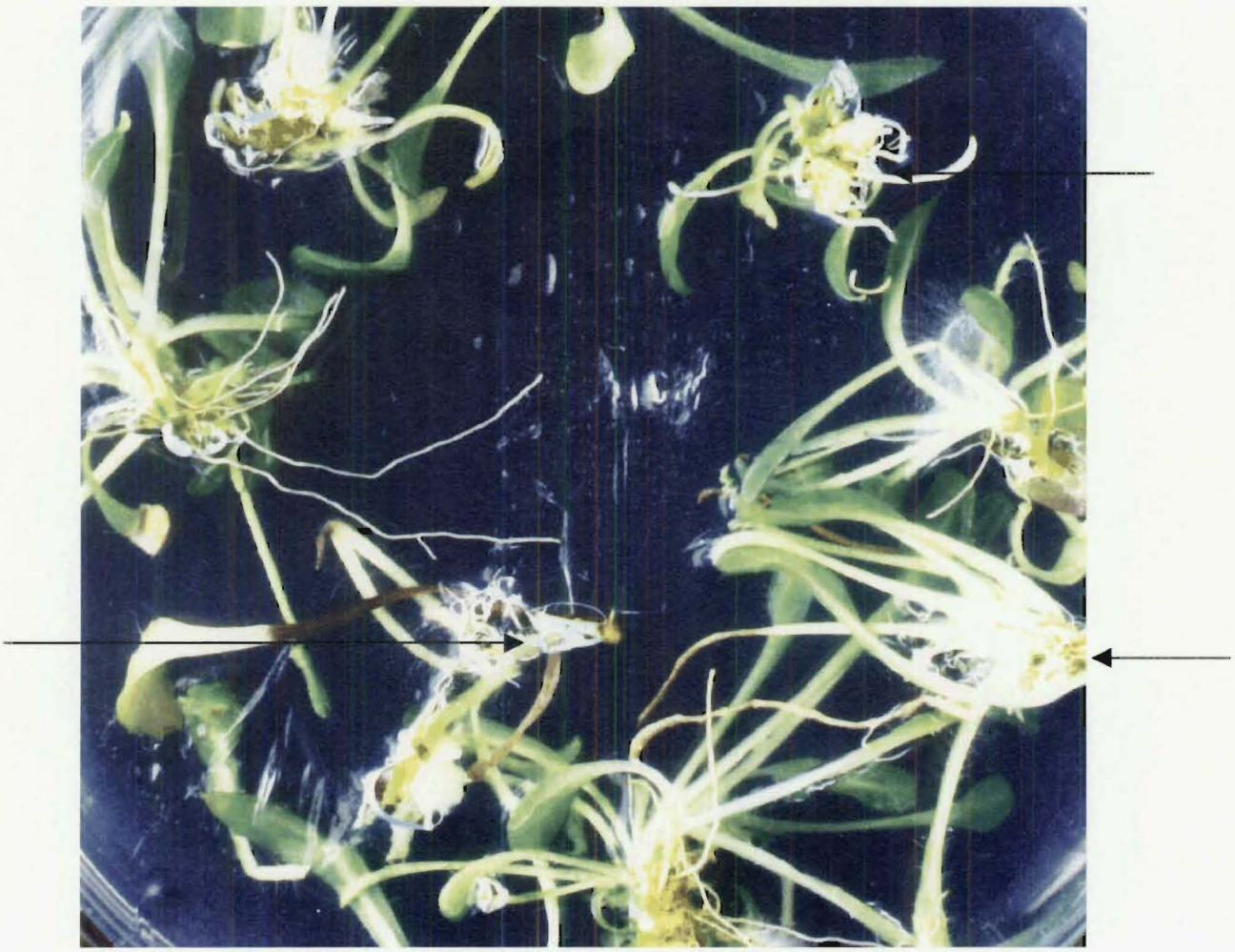


A: Tissue from plants with detectable β-glucuronidase activity.

B: Tissue from plants with no detectable β-glucuronidase activity.

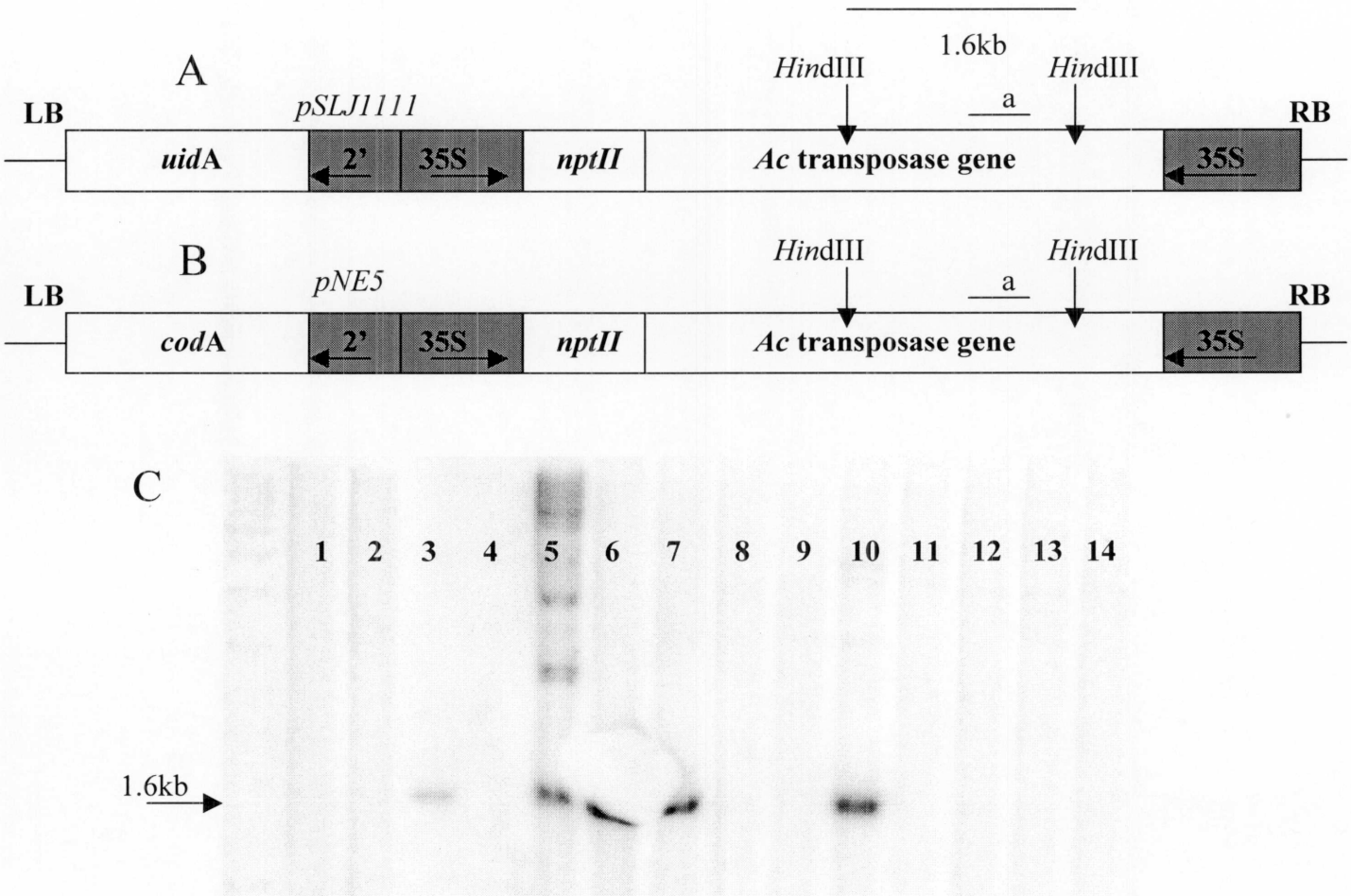
Some plants had both tissue with visible β-glucuronidase activity and tissue without visible β-glucuronidase activity(arrows).

Figure 3. 3. 10.
H. aurantiacum A3 3621 shoots regenerated after co-cultivation with *Agrobacterium tumefaciens (pNE5)* plated on HO medium supplemented with 500ug/ml 5-Flourocytosine.



Shoots that express *cod*A fail to form roots (arrows).

Figure 3. 3. 11.
Autoradiography of *Hin*dIII digested *H. aurantiacum* genomic DNA hybridised to a labelled probe homologous to the *Ac* transposase gene.



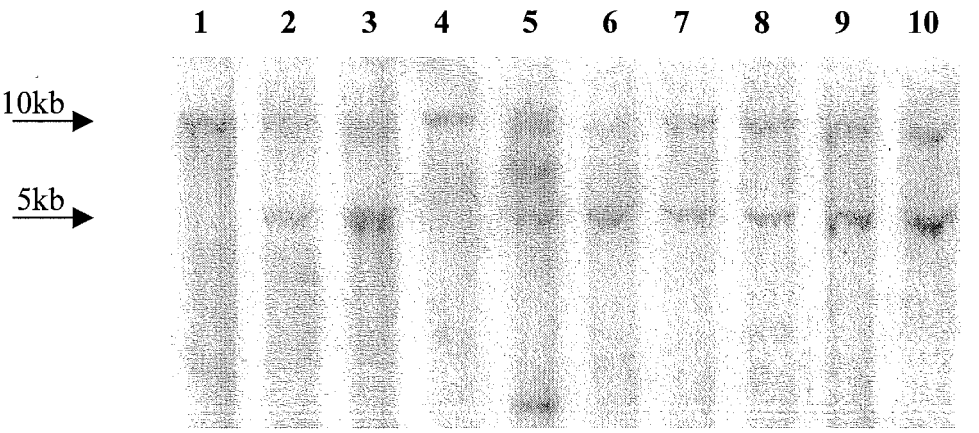
A: Map of T-DNA of *pSLJ1111*
B: Map of T-DNA of *pNE5*
C: Autoradiograph

lane 1: DNA from *H. aurantiacum* A3; lane 2: DNA from A3 3621#6 derived from A3 stably transformed with T-DNA from *pSLJ3621*; lane 3: *pSLJ1111*; lane 4: blank; lanes 5-14: DNA from spectinomycin resistant plants derived from A3 3621 co-cultivated with *pSLJ1111* or *pNE5*.

Arrow shows the expected 1.6kb *Hin*dIII transposase fragment.

*uid*A: gene for ß-glucuronidase; 2': promoter (arrow shows direction of transcription); 35S: promoter; *nptII:* kanamycin resistance gene; *Hin*dIII: restriction site for *Hin*dIII; RB: right T-DNA border; LB: left T-DNA border; a: labelled probe; *cod*A: cytosine deaminase gene.

Figure 3. 3. 12.
Autoradiograph of *Hpa*I/*Bgl*II digested *H. aurantiacum* DNA hybridised to a labelled probe homologous to the *aad*A spectinomycin resistance gene.



A 10kb *Hpa*I/*Bgl*II fragment was expected if the *Ds* element had not transposed.
A 5 kb *Hpa*I/*Bgl*II fragment was expected if the *Ds* element had transposed.

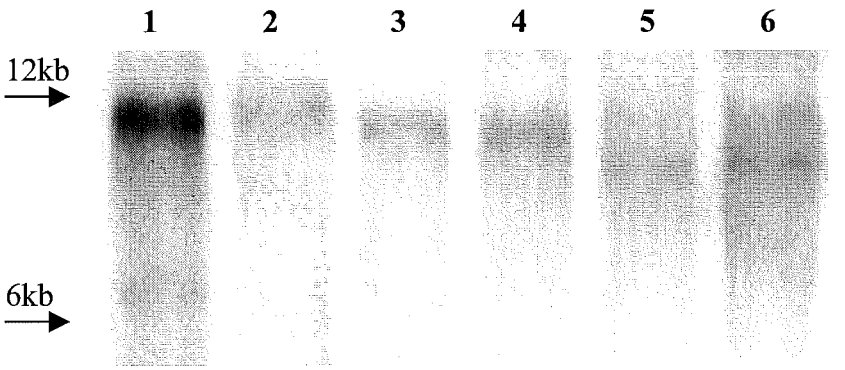
Lane1: A3 3621#6 parent plant (derived from *H. aurantiacum* A3 by transformation with *pSLJ3621*)

lanes 2-8: spectinomycin resistant plants (derived from A3 3621 plants by co-cultivation with *A. tumefaciens* (*pSLJ1111*)) that lacked *Ac* transposase sequences.

Lane 9 and 10: spectinomycin resistant plants (derived from A3 3621 plants by co-cultivation with *A. tumefaciens* (*pSLJ1111*)) that retained *Ac* transposase sequences.

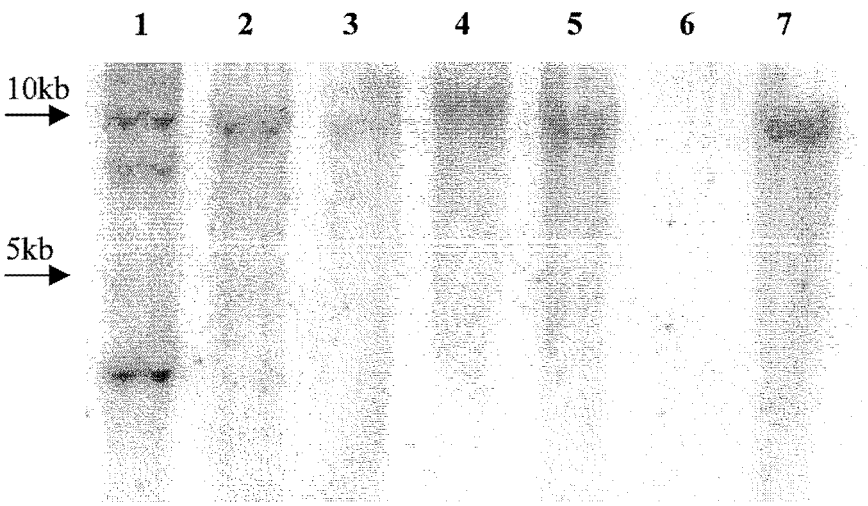
Lanes 2-4 and 6-10: DNA extracted from plants derived from A3 3621#6.
Lane 5: DNA extracted from a plant derived from another A3 3621 parent (ie. not A3 3621#6). The two additional bands in lane 5 are either due to recombination not associated with *Ds* transposition or integration of truncated *pSLJ3621* T-DNA in the original A3 3621 parental plant.

Figure 3. 3. 13.
Autoradiograph of *Hin*dIII digested *H. aurantiacum* DNA hybridised to a labelled probe homologous to the *aad*A spectinomycin resistance gene.



Lanes 1-6: DNA from individual *in vitro* A3 3621#6 plants regenerated without selection from one greenhouse-grown A3 3621#6 plant.

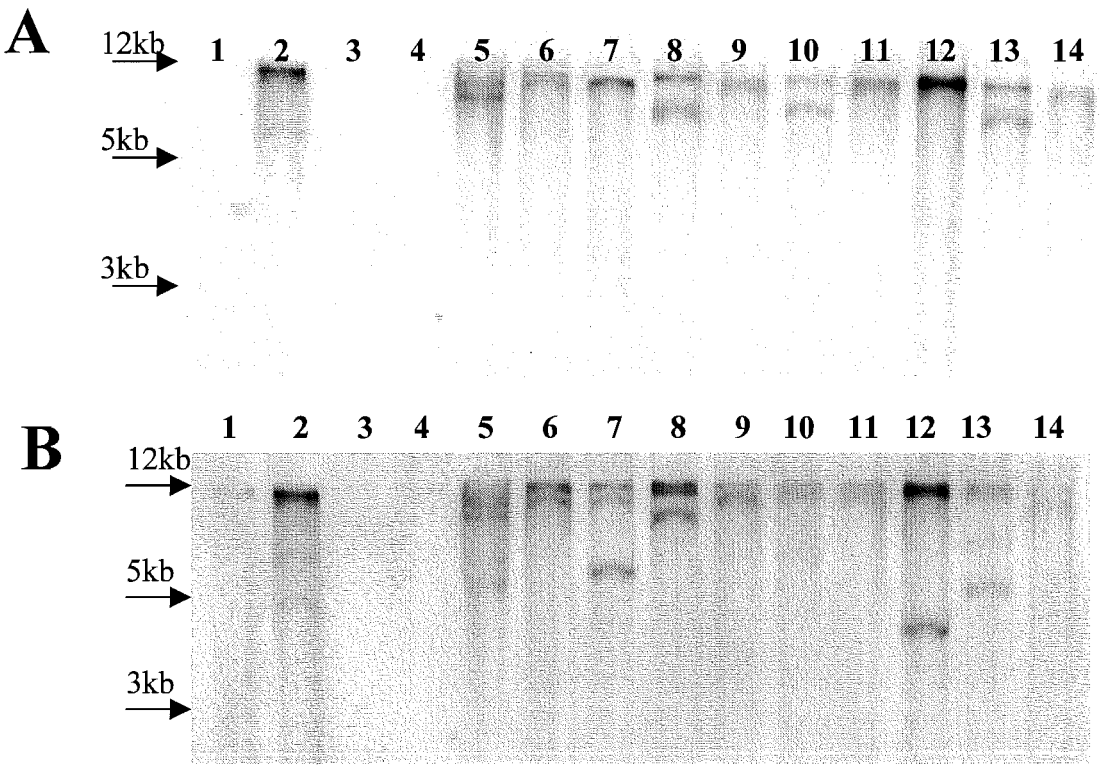
Figure 3. 3. 14.
Autoradiograph of *Hpa*I/*Bgl*II digested *H. aurantiacum* genomic DNA hybridised to a labelled probe homologous to the *aad*A spectinomycin resistance gene.



Lanes 1-7: DNA from individual *in vitro* A3 3621#6 plants regenerated without selection from one greenhouse-grown A3 3621#6 plant.

Different banding pattern between clones suggests there has been rearrangement at the *pSLJ3621* T-DNA locus during culture.

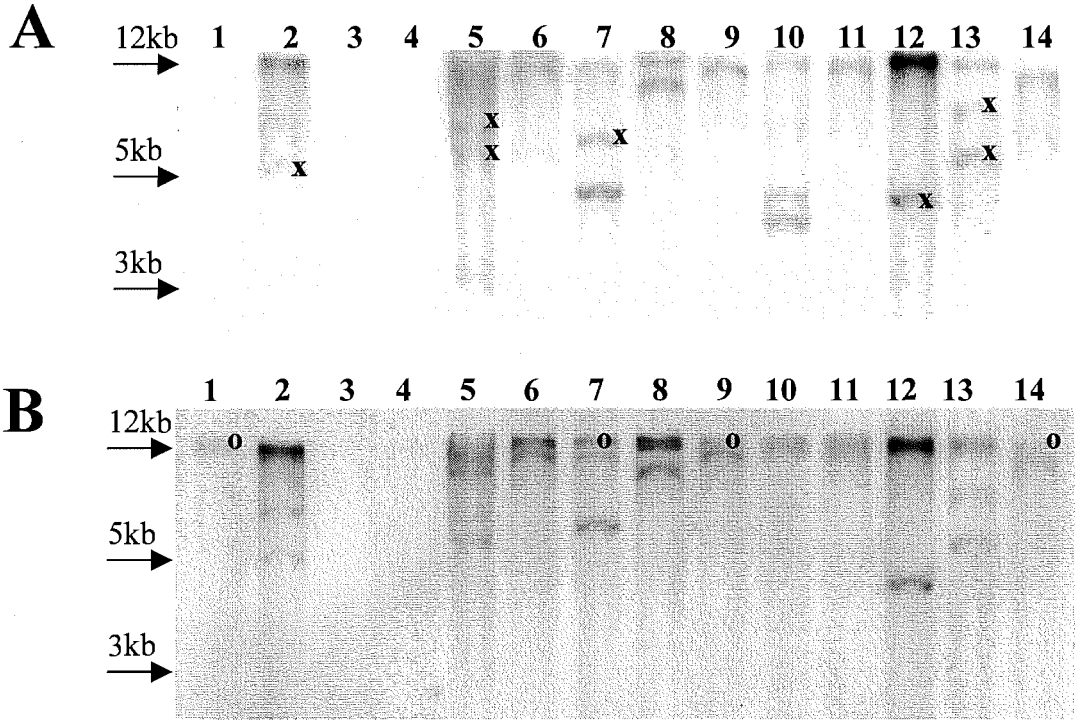
Figure 3. 3. 15.
Autoradiograph of *Hin*dIII digested *H. aurantiacum* genomic DNA hybridised to labelled probes homologous to the *bar* gene carried on the *Ds* element and the spectinomycin resistance gene(*aad*A).



A and B: Two autoradiographs taken of the same membrane. In A the membrane has been hybridised to a probe homologous to the *aad*A gene and in B the membrane has been hybridised to a probe homologous to the *bar* gene. DNA fragments that hybridise to *bar* but not *aad*A are likely due to reintegration of the *Ds* element away from the *pSLJ3621* T-DNA locus (eg. Lanes: 5, 7, 12 and 13).

lane 1: DNA from *H. aurantiacum* A3. Note faint band in B, see section 3. 3. 11 for details.
lane 2: DNA from A3 3621#6 derived from A3 by stable transformation with T-DNA from *pSLJ3621*.
lane 3: *pSLJ1111*;
lane 4: Blank
lanes 5-14: DNA from spectinomycin resistant plants derived from A3 3621 co-cultivated with *Agrobacterium tumefaciens* (*pSLJ1111* or *pNE5)*.

Figure 3. 3. 16.
Autoradiograph of *Hin*dIII digested *H. aurantiacum* genomic DNA hybridised to a labelled probe homologous to the sub-terminal region of the *Ds* element.



A and B: Two autoradiographs taken of the same membrane. In A the membrane has been hybridised to a probe homologous to the *Ds* element. In B the membrane has been hybridised to a probe homologous to the *bar* gene carried on the *Ds* element. This membrane is the same membrane as in Figure 3. 3. 14.

x: DNA fragments that hybridised to the *bar* and *Ds* probes but not the *aad*A probe (Figure 3. 3. 14) are marked on autoradiograph A with an **x**.

o: DNA fragments that hybridised to the *bar* probe but not the *Ds* probe are marked on autoradiograph B with an **o**.

Additional bands on A that are not on B are due to hybridisation of the *Ds* probe to the *Ac* fragment carried on *pSLJ1111*.

The autoradiographs demonstrate that the DNA fragments that hybridised to the *Ds* and *bar* probes but not the *aad*A probe are not due to hybridisation to plant DNA.

3. 4. Discussion

Pre-culturing *H. aurantiacum* leaf tissue on medium supplemented with plant growth regulators prior to co-cultivation with *A. tumefaciens* (carrying *pSLJ1111* T-DNA encoding β-glucuronidase) increased the frequency of cells observed with β-glucuronidase activity within the next few days. Pre-culture stimulates cell division and might enhance transgene expression by increasing the number of cells passing through S-phase during co-cultivation (49). That pre-culturing also enhanced the frequency of cells expressing β-glucuronidase after particle bombardment suggests that pre-culturing altered a step in the transformation process after DNA transfer to the plant cell. Therefore, T-DNA may typically be transferred to many more cells than it is expressed in. The expression frequency at three weeks was the same with or without pre-culturing. Therefore, high expression frequency was only delayed by not pre-culturing.

The observation that pre-culture that enhanced transient expression shortly after co-cultivation did not enhance transient expression at 3 weeks after co-cultivation is not consistent with previous research (49). Villemont et al. (49) did not observe a high expression frequency three weeks after co-cultivation of *Petunia hybrida* leaf discs with *A. tumefaciens* without at least 2 days pre-culture on medium supplemented with plant growth regulators. The most obvious difference between their transformation protocol and the protocol used here is that after 2 days co-cultivation the *P. hybrida* leaf discs were washed in a high concentration of cefotaxime (750mg/l) which possibly killed or removed the *A. tumefaciens*. Here, the *H. aurantiacum* leaf discs were placed on medium supplemented with 200mg/l timentin, which may have allowed T-DNA transfer to continue for several days.

Despite the efficiency of transient and stable transformation by co-cultivation of *H. aurantiacum* with *A. tumefaciens*, the frequency of transgene expression after particle bombardment was very low. The low frequency of detectable transient expression after bombardment limits the usefulness of this plant as a model to develop the *Ds* integration system that was to be applied to onion. The failure to recover *Ds* transposition events after particle bombardment was probably due to the low frequency of transgene expression and, in contrast to expression after co-cultivation, the predominant occurance of expression in cells from which shoots did not regenerate. A particular peculiarity of expression after

particle bombardment was that expression was often primarily observed in stomatal guard cells. Guard cells differ from surrounding epidermal cells morphologically, physiologically and even in plastid content. Any of these factors could account for the high frequency of transient expression observed in guard cells either through increased accessibility for DNA transfer or through increased expression of transferred DNA. Also, gene expression specifically concentrated in guard cells has previously been observed associated with a gene silencing phenomenon (50). Localised silencing of an endogenous gene was initiated by transfer of homologous DNA. Silencing spread through the plant body by a symplastically transmitted signal. Guard cells retained expression presumably because they were symplastically-isolated (50). Perhaps a symplastically-transmitted silencing signal is involved in suppressing expression.

Six plants in which the *Ds* element had transposed, but which lacked transposase sequences, were regenerated from A3 3621 tissue after co-cultivation with *A. tumefaciens* (*pSLJ1111* or *pNE5*). Of these six plants, one was obtained in experiment 1, and the other 5 were obtained in two subsequent experiments. In experiment 1, tissue was taken at random from the seven A3 3621 plants. Control and test tissue was taken from this pooled tissue. Control tissue was cultured, without co-cultivation, on HR medium supplemented with spectinomycin. As it is possible that co-cultivation itself could conceivably alter plant regeneration characteristics, a further control was added in subsequent experiments. In this control, the A3 3621 tissue was co-cultivated with *A. tumefaciens* lacking the binary vectors carrying the *Ac* transposase gene. In these latter experiments, tissue from each A3 3621 plant was kept separate and control tissue was taken specifically from each A3 3621 plant to eliminate the possibility that control and test tissue was taken from different plants.

In this discussion I have also included the four "control" plants discussed in section 3. 3. 9 as being derived from co-cultivation of A3 3621 tissue with *A. tumefaciens* (*pSLJ1111*). Given the results of the molecular analysis of these plants, it would have been inappropriate to treat them as control plants. Leaving them out of the discussion would not have altered the conclusions reached, but would have provided an incomplete picture of the data obtained.

After co-cultivation of A3 3621 tissue (containing the T-DNA from the *Ds* source *pSLJ3621*) with *A. tumefaciens* (containing the *Ac* transposase source *pSLJ1111*) or bombardment with the *Ac* transposase source *pSLJ1101*, some shoots were recovered that had only low level resistance to spectinomycin. These shoots could grow on HR medium supplemented with 600mg/l spectinomycin but did not form roots on HO medium supplemented with 600mg/l spectinomycin. However, they did form roots on HO medium without spectinomycin. Molecular analysis of four of these plants revealed that the *Ds* element had not transposed. Also, the autoradiograph band pattern observed after *Bgl*II/*Hpa*I digestion and hybridisation to the *aad*A probe suggested that the plants had multiple, incomplete copies of the (*pSLJ3621*) T-DNA. As the hybridisation band patterns of all four plants were identicle they might be clonally derived from tissue that possibly had low level resistance to spectinomycin prior to transfer of the *Ac* transposase gene.

The plant tissue used in this study came from seven A3 3621 plants that were separately regenerated from *H. aurantiacum* (A3) tissue after co-cultivation with *A. tumefaciens* (*pSLJ3621*). In the *Ds* excision experiment where A3 3621 tissue was bombarded with the *Ac* transposase source, and in the first *Ds* excision experiment where A3 3621 tissue was co-cultivated with *A. tumefaciens* (*pSLJ1111*), the plant tissue was taken from all seven A3 3621 plants. Shoots with low level spectinomycin resistance could be regenerated from one of the seven A3 3621 plants without transfer of the *Ac* transposase source. Tissue from this particular A3 3621 plant was probably one souce of the plants with low level spectinomycin resistance isolated in the bombardment experiment and the first co-cultivation erxperiment. In subsequent *Ds* excision experiments, tissue from two genetically and molecularly characterised A3 3621 plants were used as the *Ds* source tissue. In these subsequent experiments, shoots with low level spectinomycin resistance were still occasionally observed. Such plants did not arise after the control co-cultivations of A3 3621 tissue with *A. tumefaciens* lacking the binary vectors. Such shoots probably arose after *Ds* transposition. The inability to form roots on medium supplemented with spectinomycin was probably due to poor expression of the *aad*A gene. Poor *aad*A expression could have been caused by mutation associated with *Ds* transposition.

Transient transposase expression following transfer of an *Ac* transposase construct from *A. tumefaciens* to *H. aurantiacum* rarely resulted in transposition of the chromosomal *Ds*

element without stable integration of the transposase construct into the plant genome. 8.3% (7 out of 84) of spectinomycin resistant plants regenerated under selection for *Ds* excision did not retain *Ac* transposase sequences. Despite the lack of *Ac* transposase sequences, the *Ds* element had transposed in at least 6 of the 7 plants. This frequency of *Ds* transposition without integration of T-DNA carrying the transposase gene is higher than has previously been reported (12). Selection for transposition of a chromosomal *Ds* element following transfer of an *Ac* transposase construct from *A. tumefaciens* into potato resulted in all regenerants retaining transposase sequences (12).

Recovery of 6 regenerants with *Ds* transposition but lacking transposase sequences confirms the assumption that transient expression following T-DNA transfer can be associated with total loss of T-DNA. Proof of this conclusion has also been provided by a recent study in which transient *cre* expression mediated excision of chromosomal DNA flanked by *lox* sequences (17). In that study, plant cells containing chromosomal DNA flanked by *lox* sequences were co-cultivated with *A. tumefaciens* containing the *cre* gene within the T-DNA (T-DNA-*cre*) on the Ti plasmid. Transiently expressed *cre* recombinase could catalyse recombination at the *lox* sites resulting in excision of the intermediate DNA. After selection for cells that had precise recombination at the lox *sites* and did not express a marker gene carried on the T-DNA-*cre*, 33% (2 out of 6) of recovered cell lines lacked the *cre* gene. Similarly, Vergunst and Hooykaas (48) isolated a cell line in which *cre/lox* recombination occurred without *cre* T-DNA integration. Considering the high ratios of transient to stable expression previously reported after T-DNA transfer (24) it is surprising that in both the results reported here and by Gleave et al. (17), the proportion of recovered cells lacking T-DNA sequences (*cre* or *Ac* transposase gene) is so low. There are three possible reasons for the low proportion of cells lacking the T-DNA. Firstly, the recombination events (*Ds* transposition or *lox* recombination) might have been associated with stable T-DNA expression. Secondly, transient expression events might have been rare. Finally, expression might have been lost through gene silencing as well as T-DNA loss.

High ratios of transient to stable expression have been reported after T-DNA transfer. For example, T-DNA transfer into *Petunia* leaf discs has been estimated to result in a frequency of transient expression 1000 fold higher than the frequency of stable expression

(24). However, such high ratios of transient to stable expression are not universal. The observed frequency of transient expression in *H. aurantiacum* was no more than a few fold higher than stable expression. In two experiments no decrease in the frequency of expression was observed one month after co-cultivation. These ratios of transient to stable expression are very low compared to previous reports in other plants. It is possible that the gene tagging strategy reported here would be more effective in plant species with a higher ratio of transient to stable expression.

Another possible explanation for the low frequency of spectinomycin resistant regenerants lacking transposase sequences is that *Ds* transposition from a chromosomal locus is associated with T-DNA integration. It has previously been demonstrated that cells in S-phase and M-phase are more likely than other cells to be stably transformed by T-DNA constructs (32, 49). *Ac* and *Ds* transposition is dependent on DNA replication (9, 18, 52). Thus, cells that are predisposed to *Ds* transposition may also be predisposed to integrate the transposase gene construct. Stable transformation may also be associated with *Ds* transposition simply because the stably expressed transposase was available longer.

The low number of cells that lost the *Ac* transposase gene might limit the practicality of this gene tagging system as the *Ds* element would be unstable in cells expressing transposase. Several strategies might increase the frequency of regenerants lacking transposase sequences. DNA transfer from *A. tumefaciens* mutants lacking the omega region of the *vir*D2 gene has been reported to result in a relatively high frequency of T-DNA transfer with a greatly reduced frequency of T-DNA integration (33, 34, 42). While a role for the VirD2 protein in T-DNA integration has been disputed and is unproven (7, 47), it is possible that mutations in the *vir*D2 omega region do specifically inhibit T-DNA integration. *A. tumefaciens* strains harbouring the *vir*D2 mutation could be incorporated into this gene tagging strategy.

T-DNA might be highly protected from degradation by bound proteins when it enters the plant cell (36, 47, 55). Direct transfer of plasmid DNA free from proteins might result in a high frequency of transient transposase expression with subsequent degradation of the transposase construct without stable integration. Also, it has previously been shown that direct transfer of transposase mRNA is sufficient to cause the transposition of *Ds* elements

(28). However, experiments on direct transfer to *H. aurantiacum* have not yet produced a protocol as efficient as co-cultivation. Consequently, *Ds* transposition after direct transfer of a transposase construct or transposase mRNA to *H. aurantiacum*, has not yet been demonstrated.

Another obstacle to the development of this gene tagging strategy is presented by the high frequency of plants that did not express the T-DNA marker gene (*uidA or codA*) but had integrated the transposase gene. Enrichment for plants that did not integrate the transposase gene was inefficient because plants that did integrate the transposase gene but failed to express the *uid*A or *cod*A marker gene were not eliminated. The mechanism of loss of marker gene expression is unknown. Presumably the marker gene expression was either lost through partial T-DNA deletion or gene silencing. As the marker gene was adjacent to the left T-DNA border, small T-DNA deletions are likely to have curtailed marker gene expression without eliminating the transposase gene. Gene silencing could also have turned off marker gene expression without impeding transposase expression. No work has been undertaken to determine whether the marker gene was silenced or deleted and whether the transposase gene was expressed in the plants lacking marker gene expression.

The gene-tagging strategy described here has advantages over conventional gene tagging systems. Transient transposase expression, while sufficient for *Ds* transposition, should produce stable *Ds* loci after transposition. The one-step production of stable *Ds* loci eliminates the need to genetically manipulate regenerated plants to separate the *Ds* and transposase loci. This strategy eliminates the need to cross plants containing the *Ds* element with plants containing the transposase gene and allows gene tagging to be carried out in an isogenic background. Further research into this system is required to increase the efficiency of selecting excision events that do not coincide with stable integration of the transposase construct.

Another *Ac*/*Ds* gene tagging strategy has been developed by other groups (14, 20). In that system both elements (*Ac* transposase source and *Ds* element) are transferred into the same plant cell simultaneously (co-transformation). Transient expression of the *Ac* transposase gene results in transposition of the *Ds* element from extrachromosomal plasmid DNA and insertion into the plant genome. As the *Ds* element is mobilised by transient transposase

expression, the *Ac* transposase source does not always integrate. When the transposase source does not integrate, transposase activity will be permanently lost and the reintegrated *Ds* element will be stable (20, 45). Similar to the gene tagging strategy explored here, the co-transformation gene tagging strategy and the T-DNA gene tagging strategy (gene mutation and tagging simply through T-DNA integration) might be capable of creating mutations through repair of integration intermediates and *in vitro* culture (30). Such lesions and mutations, unlinked to the *Ds* integration site, could complicate any attempt at gene tagging. Further, a particular advantage of using *Ac/Ds* transposons for gene tagging is that the transposable element preferably transposes to sites linked to the excision site in some plant species (25, 26, 29, 43). This property of *Ac/Ds* has been successfully exploited by genetically linking the *Ds* excision locus to the target site, thus maximising the probability of tagging the gene of interest once the transposon is mobilised (eg. see: 6). Co-transformation and T-DNA tagging do not allow selection of plants with a stable *Ds* excision locus linked to the target gene prior to mobilising the *Ds* element.

In order to evaluate the potential usefulness of the various available gene tagging systems, the relative frequency of three possible causes of phenotypic variation need to be considered. These are: variation due to tissue culture and transformation; variation due to stable *Ds* insertion; and variation due to *Ds* insertion and re-excision (secondary transposition). We know that the frequency of secondary transposition can be much higher than the primary transposition frequency (35). However, mutation due to transformation and tissue culture may also be high. Marton et al. (30) reported a 50% decline in plating efficiency of haploid tobacco protoplasts they attributed primarily to mutations caused by the transient presence of T-DNA in the plant cell after co-cultivation with a virulent *A. tumefaciens* strain. *In vivo* transformation strategies (eg. gene transfer to germ cells within the plant) and/or transfer of mRNA rather than DNA might eliminate the mutagenic effect of transformation. Combining such transformation approaches with the gene tagging strategy examined here might produce an ideal method for gene tagging in some plants.

3. 5. References

1. Altmann T, Schmidt R, Willmitzer L: Establishment of a gene tagging system in *Arabidopsis thaliana* based on the maize transposable element *Ac*. Theoretical and Applied Genetics 84: 371-383 (1992).

2. Bancroft I, Bhatt A M, Sjodin C, Scofield S, Jones JDG, Dean C: Development of an efficient two-element transposon tagging system in *Arabidopsis thaliana*. Molecular and General Genetics 233: 449-461 (1992).

3. Becker D, Lutticke R, Li M, Starlinger P: Control of excision frequency of maize transposable element *Ds* in *Petunia* protoplasts. Proceedings of the National Academy of Science USA 89: 5552-5556 (1992).

4. Bicknell RA: Micropropagation of *Hieracium aurantiacum*. Plant Cell, Tissue and Organ Culture 37: 197-199 (1994).

5. Bicknell RA, Borst NK: *Agrobacterium*-mediated transformation of *Hieracium aurantiacum*. International Journal of Plant Science 155(4): 467-470 (1994).

6. Bishop GJ, Harrison K, Jones JDG: The tomato dwarf gene isolated by heterologous transposon tagging encodes the first member of a new cytochrome P450 family. The Plant Cell 8: 959-969 (1996).

7. Bravo-Angel AM, Hohn B, Tinland B: The Omega sequence of VirD2 is important but not essential for efficient transfer of T-DNA by *Agrobacterium tumefaciens*. Molecular Plant-Microbe Interactions 11(1): 57-63 (1998).

8. Carroll B, Klimyuk V, Thomas CM, Bishop GJ, Harrison K, Scofield S, Jones J: Germinal transpositions of the maize element *Dissociation* from T-DNA loci in tomato. Genetics 139: 407-420 (1995).

9. Chen J, Greenblatt IM, Dellaporta SL: Molecular analysis of *Ac* transposition and DNA replication. Genetics 130: 665-676 (1992).

10. De Buck S, Jacobs A, Van Montagu M, Depicker A: *Agrobacterium tumefaciens* transformation and cotransformation frequencies of *Arabidopsis thaliana* root explants and tobacco protoplasts. Molecular Plant-Microbe Interactions 11(6): 449-457 (1998).

11. Eady C, Lindsey K, Twell D: Differential activation and conserved vegetative cell-specific activity of a late pollen promoter in species with bicellular and tricellular pollen. The Plant Journal 5: 543-550 (1994).

12. El-Kharbotly A, Jacobs JME, Hekkert B, Jacobsen E, Ramanna MS, Stiekema WJ, Pereira A: Localisation of *Ds*-transposon containing T-DNA inserts in the diploid transgenic potato: linkage to the R1 resistance gene against *Phytophthora infestans* (Mont.) de Bary. Genome 39: 249-257 (1996).

13. Finnegan E J, Lawrence G J, Dennis E S, Ellis J G: Behaviour of modified *Ac* elements in flax callus and regenerated plants. Plant Molecular Biology 22: 625-633 (1993).

14. Fitzmaurice W P, Lehman L V, Thompson W F, Wernsman E A, Conkling M A: Development and characterization of a generalized gene tagging system for higher plants using an engineered maize transposon *Ac*. Plant Molecular Biology 20: 177-198 (1992).

15. Gallego ME, Sirand-Pugnet P, White CI: Positive-negative selection and T-DNA stability in *Arabidopsis* transformation. Plant Molecular Biology 39: 83-93 (1999).

16. Gleave AP: A versatile binary vector system with a T-DNA organisational structure conducive to efficient integration of cloned DNA into the plant genome. Plant Molecular Biology 20: 1203-1207 (1992).

17. Gleave AP, Mitra DS, Mudge SR, Morris BAM: Selectable marker-free transgenic plants without sexual crossing: transient expression of *cre* recombinase and the use of a conditional lethal dominant gene. Plant Molecular Biology 40: 223-235 (1999).

18. Greenblatt IM, Brink RA: Twin mutations in medium variegated pericarp maize. Genetics 47: 489-501 (1961).

19. Houba-Herin N, Becker D, Post A, Larondelle Y, Starlinger P: Excision of a *Ds*-like maize transposable element (*Ac*) in a transient assay in Petunia is enhanced by a truncated coding region of the transposable element *Ac*. Molecular and General Genetics 224: 17-23 (1990).

20. Houba-Herin N, Domin M, Leprince A-S: Some features about transposition of the maize element *Dissociation* in *Nicotiana plumbaginifolia*. Genetica 93: 41-48 (1994).

21. Houba-Herin N, Domin M, Pedron J: Transposition of a *Ds* element from a plasmid into the plant genome in *Nicotiana plumbaginifolia* protoplast-derived cells. The Plant Journal 6: 55-66 (1994).

22. Izawa T, Ohnishi T, Nakano T, Ishida N, Enoki H, Hashimoto H, Itoh K, Terada R, Wu C, Miyazaki C, Endo T, Iida S, Shimamoto K: Transposon tagging in rice. Plant Molecular Biology 35: 219-229 (1997).

23. James D W, Lim E, Keller J, Plooy I, Ralston E, Dooner H K: Directed tagging of the *Arabidopsis* fatty acid elongation 1 (FAE1) gene with the maize transposon *Activator*. The Plant Cell 7: 309-319 (1995).

24. Janssen B-J, Gardner RC: Localized transient expression of GUS in leaf discs following cocultivation with *Agrobacterium*. Plant Molecular Biology 14: 61-72 (1989).

25. Jones JDG, Carland F, Lim E, Ralston E, Dooner HK: Preferential transposition of the maize element *Activator* to linked chromosomal locations in tobacco. Plant Cell 2: 701-707 (1990).

26. Keller J, Lim E, Dooner H K: Preferential transposition of *Ac* to linked sites in *Arabidopsis*. Theoretical and Applied Genetics 86: 585-588 (1993).

27. Laufs J, Wirtz U, Kammann M, Matzeit V, Schaefer S, Schell J, Czernilofsky AP, Baker B, Gronenborn B: Wheat Dwarf Virus *Ac/Ds* vectors: expression and excision of transposable elements introduced into various cereals by a viral replicon. Proceedings of the National Academy of Science USA 87: 7752-7756 (1990).

28. Lebel EG, Masson J, Bogucki A, Paszkowski J: Transposable elements as plant transformation vectors for long stretches of foreign DNA. Theoretical and Applied Genetics 91: 899-906 (1995).

29. Machida C, Onouchi H, Koizumi J, Hamada S, Semiarti E, Torikai S, Machida Y: Characterization of the transposition pattern of the *Ac* element in *Arabidopsis thaliana* using endonuclease I-SceI. Proceedings of the National Academy of Science USA 94: 8675-8680 (1997).

30. Marton L, Hrouda M, Pecsvaradi A, Czako M: T-DNA-insert-independent mutations induced in transformed plant cells during *Agrobacterium* co-cultivation. Transgenic Research 3: 317-325 (1994).

31. McElroy D, Louwerse JD, McElroy SM, Lemaux PG: Development of a simple transient assay for *Ac/Ds* activity in cells of intact barley tissue. The Plant Journal 11: 157-165 (1997).

32. Meyer P, Walgenbach E, Bussmann K, Hombrecher G, Saedler H: Synchronized tobacco protoplasts are efficiently transformed by DNA. Molecular and General Genetics 201: 513-518 (1985).

33. Mysore KS, Bassuner B, Deng X, Darbinian NS, Motchoulski A, Ream W, Gelvin SB: Role of the *Agrobacterium tumefaciens* VirD2 protein in T-DNA transfer and integration. Molecular Plant-Microbe Interactions 11(7): 668-683 (1998).

34. Narasimhulu SB, Deng X, Sarria R, Gelvin SB: Early transcription of *Agrobacterium* T-DNA genes in tobacco and maize. The Plant Cell 8: 873-886 (1996).

35. Robbins TP, Jenkin M, Courtney-Gutterson N: Enhanced Frequency of Transposition of the Maize Transposable element *Activator* following excision from T-DNA in *Petunia Hybrida*. Molecular and General Genetics 244: 491-500 (1994).

36. Rossi L, Hohn B, Tinland B: Integration of complete transferred DNA units is dependent on the activity of virulence E2 protein of *Agrobacterium tumefaciens*. Proceedings of the National Academy of Science USA 93: 126-130 (1996).

37. Sambrook J, Fritsch EF, Maniatis T: Molecular cloning. A laboratory manual. Ed. 2. Cold Spring Harbour Laboratory Press, (1989).

38. Scofield SR, Harrison K, Nurrish SJ, Jones JDG: Promoter fusions to the *Activator* transposase gene confer distinct patterns of *Dissociation* excision in tobacco cotyledons. Plant Cell 4: 573-582 (1992).

39. Scott L, LaFoe D, Weil CF: Adjacent sequences influence DNA repair accompanying transposon excision in maize. Genetics 142: 237-246 (1996).

40. Shen W-H, Ramos C, Hohn B: Excision of *Ds1* from the genome of maize streak virus in response to different transposase-encoding genes. Plant Molecular Biology 36: 387-392 (1998).

41. Shimamoto K, Miyazaki C, Hashimoto H, Izawa T, Itoh K, Terada R, Inagaki Y, Iida S: Trans-activation and stable integration of the maize transposable element *Ds* cotransfected with the *Ac* transposase gene in transgenic rice plants. Molecular and General Genetics 239: 354-360 (1993).

42. Shurvinton CE, Hodges L, Ream W: A nuclear localisation signal and the C-terminal omega sequence in the *Agrobacterium tumefaciens* VirD2 endonuclease are important for tumour formation. Proceedings of the National Academy of Science USA 89: 11837-11841 (1992).

43. Smith D, Yanai Y, Liu Y-G, Isiguro S, Okada K, Shibata D, Whittier RF, Fedoroff NV: Characterisation and mapping of *Ds*-GUS-T-DNA lines for targeted insertional mutagenesis. The Plant Journal 10: 721-732 (1996).

44. Stougard J, Substrate-dependent negative selection in plants using a bacterial cytosine deaminase gene. The Plant Journal 3 (5) 755-761.

45. Sugimoto K, Otsuki Y, Saji S, Hirochika H: Transposition of the maize *Ds* element from a viral vector to the rice genome. The Plant Journal 5: 863-871 (1994).

46. Swinburne J, Balcells L, Scofield SR, Jones JDG, Coupland G: Elevated levels of *Activator* transposase mRNA are associated with high frequencies of *Dissociation* excision in *Arabidopsis*. The Plant Cell 4: 583-595 (1992).

47. Tindland B, Schoumacher F, Gloeckler V, Bravo-Angel AM, Hohn B: The *Agrobacterium tumefaciens* virulence D2 protein is responsible for precise integration of T-DNA into the plant genome. The EMBO Journal 14(14): 3585-3595 (1995).

48. Vergunst AC, Hooykaas PJJ: *Cre/lox*-mediated site-specific integration of *Agrobacterium* T-DNA in *Arabidopsis thaliana* by transient expression of *cre*. Plant Molecular Biology 38: 393-406 (1998).

49. Villemont E, Dubios F, Sangwan RS, Vasseur G, Bourgeois Y, Sandwan- Norreel BS: Role of the host cell cycle in the *Agrobacterium*-mediated genetic transformation of *Petunia*: evidence of an S-phase control mechanism for T-DNA transfer. Planta 201: 160-172 (1997).

50. Voinnet O, Vain P, Angell S, Baulcombe DC: Systemic spread of sequence-specific transgene RNA degradation in plants is initiated by localized introduction of ectopic promoterless DNA. Cell 95: 177-187 (1998).

51. Weld R, Heinemann J, Eady C: Transient GFP expression in *Nicotiana plumbaginifolia* suspension cells following co-cultivation with *Agrobacterium tumefaciens*: the role of gene silencing, cell death and T-DNA loss. (submitted).

52. Wirtz U, Osborne B, Baker B: *Ds* excision from extrachromosomal geminivirus vector DNA is coupled to vector DNA replication in maize. The Plant Journal 11(1): 125-135 (1997).

53. Yang CH, Carroll B, Scofield S, Jones J, Michelmore R: Transactivation of *Ds* elements in plants of lettuce (*Lactuca sativa*). Molecular and General Genetics 241: 389-398 (1993).

54. Yoshioka Y, Takahashi Y, Matsuoka K, Nakamura K, Koizumi J, Kojima M, Machida Y: Transient gene expression in plant cells mediated by *Agrobacterium tumefaciens*: application for the analysis of virulence loci. Plant Cell Physiology 37(6): 782-789 (1996).

55. Zupan J, Zambryski P: The *Agrobacterium* DNA transfer complex. Critical Reviews in Plant Sciences 16(3): 279-295 (1997).

Chapter 4.

Transient GFP Expression in *Nicotiana plumbaginifolia* Suspension Cells Following Co-cultivation with *Agrobacterium tumefaciens*: the Role of Gene Silencing, Cell Death and T-DNA Loss.

4. 1. Introduction

Procedures based on the natural transformation system of *Agrobacterium tumefaciens* are widely used to transfer genes into plant cells. After co-cultivation of plant cells with *A. tumefaciens*, T-DNA expression and the frequency of plant cells expressing the transgene increases rapidly, peaks within a few days and then declines over subsequent weeks (14, 24, 27, 46). This temporal profile of transgene expression led to the conclusion that there was transcription from unstable extrachromosomal copies of the T-DNA that were not maintained in the plant cell (14, 46). As a consequence of this conclusion, it has become widely assumed that T-DNA integration into the plant genome is a major factor limiting the frequency of "stable" transformation events following *A. tumefaciens*-mediated gene transfer (3, 27, 39).

A substantial body of circumstantial evidence supports the current model of transient expression from T-DNA. The rate of loss of expression after DNA transfer roughly correlates with the rate of loss of extrachromosomal DNA in plant cells (43, 14, 27, 46). The temporal profile of transient expression after T-DNA transfer was precisely the same as transient expression after direct transfer of non-replicating, double-stranded plasmid DNA (14). Further, tissue lacking T-DNA was detected after shoot regeneration from samples of cells that had transiently or stably expressed a T-DNA (3, 8, 41).

Transient expression is often used in the optimisation of transformation protocols as an indicator of gene transfer and expression efficiency (5, 7, 13, 15, 16, 17, 22). Understanding the molecular determinants of transient expression is important, particularly where high levels of transient expression do not lead to high frequencies of stable transformation (22). Alternative interpretations of transient expression suggest contrasting

strategies for enhancing stable transformation (eg. increasing transfer in order to increase integration or decreasing transfer to avoid gene silencing).

Our research into onion transformation floundered when the tissue culture and DNA transfer system, optimized for transient expression and plant regeneration, did not readily produce stable transformants. A DNA integration system (*Ds* transposition) was sought to overcome the low rate of conversion of transient expression into stable expression. The assumption behind that strategy was that transient expression was lost through failure to integrate transferred DNA. Until now, no study has isolated and analysed a random sample of transiently expressing plant cells to quantify the causes of loss of expression. The objective of this study was to determine to what extent transient expression, in our model system, was caused by total loss of T-DNA, gene silencing or cell death.

4. 2. Materials and Methods

4. 2. 1. Cell growth and co-cultivation conditions.

N. plumbaginifolia suspension cells were maintained with twice weekly subculture (5ml/40ml dilution) in CS-V medium (as per Lorz (18) but with 0.05mg/l kinetin and pH 5.8) in 125 ml flasks on a rotating platform (166 rpm) at 22-26^{0}C (16 hr light/8 hr dark). *A. tumefaciens* LBA4404 containing the binary vector *pBINm-gfp5-ER* (32) was incubated for approximately 12 hours at 28^{0}C in LB medium (36) with 100ug/ml kanamycin. 4 ml *A. tumefaciens* saturated culture was diluted into 50ml fresh LB medium containing 50mM acetosyringone. After 4 hr incubation, cells from 5-10 ml were pelleted (3000xg, 10 minutes), resuspended in 5ml 3-4 day old *N. plumbaginifolia* suspension cell culture and co-cultivated without shaking, for 1hr at 28^{0}C in 9cm petri dishes. Cells were pelleted (200xg, 2 minutes), liquid decanted and the cells were transfered to filter paper on solid CS-V medium for 3 days co-cultivation.

4. 2. 2. Isolation and culture of transiently expressing cells.

After 3 days co-cultivation, approximately 50ul of cells were transferred to 500ul protoplast-isolation enzyme mix (cellulase 0.9% (Onozuka R-10, Yakult Honsha),

hemicellulase 0.45% (Sigma), driselase 0.45% (Kyowa Hakko Kogyo), MES 0.15%, sorbitol 9%, pH 5.5 with NaOH). Cells were incubated in enzyme mix in the dark for approximately 5 hr (22-26^{0}C). 1 ml ASW (311mM NaCl, 6.9mM KCl, 18.8mM $MgSO_4$. $7H_2O$, 16.7mM $MgCl_2$. $6H_2O$, 6.8mM $CaCl_2$. $2H_2O$, 1.75mM $NaHCO_3$, 10mM MES, pH 6.0) was added and protoplasts were pelleted (80-100xg, 3 minutes). Liquid was removed and protoplasts were washed in 1.5ml ASW and resuspended in 1ml CS-V medium (with 0.4M sucrose). Protoplasts were diluted in CS-V medium (with 0.4M sucrose, 1% low melting point agarose (Life Technologies Inc."Ultra Pure")) at approximately 2 x 10^5 cells per litre. 1ml of protoplast culture was spread thinly over the bottom of each 9cm petri dish. This thin layer was allowed to set and was then overlaid with 4ml CS-V medium (0.4M sucrose, 1% low melting point agarose). Once set, the solid CS-V medium was overlaid with 4ml of liquid CS-V (0.4M sucrose). To this liquid layer was added 100µl *N.plumbaginifolia* cell suspension culture as feeder cells and timentin to a final concentration of 200mg/l. Protoplasts were cultured at 22-26^{0}C in the dark. The feeder cell layer was diluted weekly (or more frequently if required to control the density of feeder cells) by replacement of 3ml suspension culture with 3ml fresh CS-V medium (0.4M sucrose and 200mg/l timentin initially then over the course of several weeks the concentration of sucrose and timentin was diminished). Care was taken to remove feeder cells that aggregated as solid surface growth. After 4 days culture, protoplasts were observed with a fluorescent microscope and well isolated, GFP expressing cells were marked by cuts to the under surface of the petri dishes. Once calli were several mm in diameter, the feeder layer was removed, and the solid medium was washed several times with liquid CS-V medium to remove feeder cells. All liquid was removed and calli were cultured until they were approximately 5mm diameter at which stage they were isolated and transferred onto fresh CS-V solid medium. Thereafter the cell lines were maintained by weekly subculture to fresh CS-V medium.

4. 2. 3. Measurement of callus diameter

The growing calli were measured *in situ* by the gradations on the microscope stage. One measurement across each callus was made at the widest point in the orientation in which they happened to lie.

4. 2. 4. DNA extraction for PCR and Southern Blot analysis.

Approximately 300µl *N.plumbaginifolia* callus cells frozen in liquid nitrogen were ground in 600µl urea extraction buffer (urea 42%, 0.3M NaOH, 0.1M Tris-HCl pH 8.0, 0.02M EDTA pH 8.0, Sarcosine 20%). DNA was purified by standard phenol/chloroform extraction, isopropanol precipitation, and desalted in 70% ethanol (36). RNA was digested in RNAseA for 60 minutes at 60^0C. All DNA samples were quantified by ethidium bromide staining and comparison to a standard (lambda DNA digested with *Hin*dIII) of known concentration.

4. 2. 5. Southern blot analyses.

DNA (10ug) was digested with *Hin*dIII (Boehringer Mannheim) and size-fractionated by electrophoresis in 1% agarose gels in Tris-borate-EDTA (TBE) buffer, transferred to nylon membrane (BioRad " Zeta probe" or Qiagen "Qiabrane") and hybridized to ^{32}P-dCTP-labelled probes at 65^0C for 12-24 hr. ^{32}P-labelling was done with a random priming kit (Amersham). The probe for the T-DNA was an 820bp fragment of *pBINm-gfp5-ER* amplified by PCR with *Taq* polymerase (Boehringer Mannheim) using primers flanking the *gfp* gene (5'-ACG TCT CGA GGA TCC AAG GAG ATA TAA CA-3' and 5'-ACG TCT CGA GCT CTT AAA GCT CAT CAT G-3'). These PCR-generated probe fragments were purified on a 0.7% agarose gel and extracted with the Qiaquick gel extraction kit (Qiagen). Restriction digestion, ^{32}P-labelling, PCR, and probe isolation were done according to the manufacturer's instructions. Final washing was performed in 1 X SSC, 0.1% SDS or 0.5 X SSC, 0.1% SDS at 65^0C. Hybridisation patterns were visualised by phosphor imaging on a Storm 840 laser scanner (Molecular Dynamics).

4. 2. 6. Analysis for DNA methylation.

The response of transient expressers to demethylation was tested. *N. plumbaginifolia* cells were transferred to solid CS-V medium supplemented with 5-azacytidine (0mM, 0.2mM, 0.5mM, 0.8mM, 1.0mM, and 5.0mM). After 3 or 14 days, the cells were returned to CS-V medium with no 5-azacytidine. GFP activity was monitored by observation using a fluorescent microscope. A direct assay of DNA methylation was also used. In this assay

genomic DNA was extracted and quantified as described above. 100-500ng from each sample was digested with *Sau*3A1, digested with *Nde*II, or incubated without restriction enzymes. After 3-12 hour digestion, DNA was precipitated in isopropanol and resuspended in 10mM Tris.Cl pH 8.0. After dilution in ddH_2O, PCR (as described below) was used to amplify the *gfp* gene fragment. 10µl from each reaction was size fractionated by electrophoresis on a 0.7% agarose gel and visualised under UV light after staining with ethidium bromide.

4. 2. 7. PCR analyses

PCR was performed on approximately 100ng total genomic DNA in a Techne Thermocycler PHC-3 with 0.625 units *Taq* polymerase (Boehringer Mannheim) in a total volume of 25µl. Amplification of the *gfp* gene (primers: 5'-ACG TCT CGA GGA TCC AAG GAG ATA TAA CA and 5'-ACG TCT CGA GCT CTT AAA GCT CAT CAT) was for 35 cycles of 1 minute at 94^0C, 1 minute at 58^0C, 1 minute at 72^0C. The 35 amplification cycles were preceded by 1 cycle for 1 minute at 94^0C and followed by 1 cycle of 72^0C for 8 minutes. Amplification of the *vir*G gene using primers GMT24 (5'-GCG GTA GCC GAC AG-3') and GMT25 (5'-GCG TCA AAG AAA TA-3') used the same programme as amplification of the *gfp* gene except that annealing was at 42^0C. Sensitivities of the *vir*G and *gfp* PCRs were compared using DNA extracted (as per DNA extraction method described above) from a mixture of plant cells and *A. tumefaciens* cells carrying the *pBINm-gfp5-ER* vector.

4. 2. 8. Statistical Analysis.

The relationship between time of protoplast first division and stable T-DNA integration for 9 transient expressers from experiment 2 (figure 4. 3. 9) was analysed by probit analysis (33). Only transient expressers were analysed. The percentage of plants showing transgenic loci for each time of first division was modelled with a logistic curve, with 100% of cells retaining *gfp* sequences at the maximum, declining to 0% of cells retaining *gfp* sequences. The curve was described by the equation:

$$\% \text{ with loci} = \frac{100}{1 + e^{\text{slope} * (\text{time} - T50)}}.$$

Where slope is related to the steepness of the curve at time = T50, and T50 is the time where only half the cells produced loci. The analysis included a weighting for the number of cells dividing at each time. The curve was tested to see whether it described the data significantly better than fitting a constant value (ie, no significant changes with time). The analysis was carried out by Crop and Food biostatistician Ruth Butler using Genstat 5.

4. 3. Results

4. 3. 1. Transient *gfp* expression

N. plumbaginifolia suspension cells were co-cultivated with *A. tumefaciens* LBA4404 containing the binary vector *pBINm-gfp5-ER* (9). *pBINm-gfp5-ER* encodes the green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* as a visual marker. 4 days after co-cultivation, the protoplasts were embedded in low melting point agarose (Figure 4. 3. 1 and Figure 4. 3. 2). Individual GFP-expressing protoplasts were marked and cultured without selection (Figure 4. 3. 3 and Figure 4. 3. 4). 4 weeks later, the resulting calli were again screened for GFP expression (Figure 4. 3. 5). GFP activity was observed in 10-20% of the protoplasts within one week from co-cultivation. Over three experiments, 50% of growing calli had lost visible GFP activity by 4 weeks post inoculation (58%, 54% and 32% in experiments 1-3 respectively). Only calli completely lacking visible GFP activity at 4 weeks were recorded as being transient expressers. However, some stably expressing calli contained some cells without GFP activity at 4 weeks and nearly all regularly produced sectors with no GFP activity during subsequent culture, and a few "stable" expressers eventually lost all GFP activity (Figure 4. 3. 6). A sample of both transiently and stably expressing calli were recovered and maintained as cultured cell lines.

4. 3. 2. Cell death

In three separate experiments, the majority of GFP expressing cells died (as judged by cessation of cytoplasmic streaming and cell membrane collapse) shortly after plating (Table 4. 3. 1). Thus, in this system, the transient nature of GFP expression was principally associated with cell death. To test whether the high mortality of cells was specifically

associated with T-DNA transfer, the mortality rate of protoplasts expressing GFP shortly after co-cultivation was compared with protoplasts from the same co-cultivation experiment that did not express GFP. In three experiments no difference in average mortality was found between healthy cells that had expressed GFP 4 days after co-cultivation and healthy cells that had not expressed GFP (Table 4. 3. 1). In experiments to optimise protoplast culture without co-cultivation with *A. tumefaciens*, mortality rates of 70-100% were regularly observed (data not shown). Therefore, the high frequency of cell death shortly after plating is likely to be primarily a result of stress associated with cell manipulation and natural cell turnover rather than a direct consequence of T-DNA transfer.

Table 4. 3. 1: Frequency of cell death following co-cultivation, isolation and plating of *N. plumbaginifolia* suspension cells.

Experiment	**Protoplasts expressing GFP 4 days post inoculation**		**Protoplasts not expressing GFP 4 days post inoculation**	
	Proportion dead[1]	**Sample size**	**Proportion dead[1]**	**Sample size**
1	67%	46	NR[2]	
2	70%	103	70%	69
3	57%	116	50%	28
Overall mortality		**64%**		**64%**

1: Proportion dead = the proportion of isolated protoplasts with cell membrane collapse and that lacked cytoplasmic streaming two weeks after co-cultivation.

2: NR = Not Recorded

4. 3. 3. T-DNA loss.

Some calli were lost through contamination and smaller calli failed to survive transfer to fresh medium. However, 17 transiently expressing calli and 33 stably expressing calli were isolated. The 17 transient expressers and 11 of the 33 stably expressing cell lines were tested for the presence of the T-DNA by PCR amplification using primers specific to the *gfp* gene (eg. Figure 4. 3. 7). PCR detected *gfp* sequences in 10 of the 17 transient expressers and all of the 11 stable expressers. To test that the positive PCR results were not due to contamination of the transiently expressing cell lines by *A. tumefaciens*, primers specific to the Ti plasmid *vir*G gene were substituted for the *gfp* primers and the PCR assay was repeated. No *vir*G sequences were detected in any transiently expressing cell lines. The PCR assay for *virG* sequences and the PCR assay for *gfp* sequences were compared to test that the different results obtained with the two assays were not due to differences in assay sensitivities. DNA was extracted from a mixture of untransformed *N. plumbaginifolia* cells and the *A. tumefaciens* strain used in this study by the same method as used to extract plant genomic DNA for PCR. PCR amplification of *virG* and *gfp* sequences was then attempted from dilution series of the DNA sample. The PCR for *gfp* sequences could amplify a product from DNA 100 times less concentrated than the lowest DNA concentration at which *virG* sequences were detectable. This experiment was repeated with the same result. To test that *gfp* sequences amplified from DNA extracted from transient expressers were not from *A. tumefaciens*, a dilution series of DNA from 4 transient expressers was used as the template for PCR amplification of *virG* and *gfp* sequences. *vir*G sequences were not detectable at any DNA concentration. *gfp* sequences could be amplified from DNA at a concentration 1000 times lower than the highest DNA concentration used in the *vir*G PCR assay. *gfp* sequences could be amplified from DNA at the higest concentration used in the *virG* PCR assay and there was no indication that PCR amplification was inhibited at that DNA concentration. It is most likely that the *gfp* sequences amplified by PCR are from T-DNA integrated into plant DNA and not due to contaminating *A.tumefaciens*.

The integration of *gfp* sequences in plant DNA was tested by Southern analysis of all 17 transient expressers and 11 stable expressers (Figure 4. 3. 8). For this analysis, plant DNA was digested with *Hin*dIII (*pBINm-gfp5-ER* has a single *Hin*dIII recognition site). The average number of bands from transiently expressing cell lines was essentially the same as

that from the stably expressing cell lines, suggesting there was not an extreme difference in the number of T-DNA integration loci. However, of the 10 transient expressers carrying the *gfp* gene, 7 contained a distinctive, 5-7kb band approximately the size of the T-DNA (Figure 4. 3. 8). The size and intensity of this band suggests it represents tandem T-DNA integrations. Tandem integrations have previously been reported to be associated with gene silencing (reviewed in 6). Prior to Southern analysis, the cell lines were cultured for several weeks with weekly sub-culture. Over this period, 4 of the 33 "stably" expressing cell lines lost all visible GFP activity. Southern analysis was used to test for the presence of the *gfp* sequence in 3 of these cell lines. 2 of the cell lines lacked *gfp* sequences and 1 had multiple bands including the high copy number 5-7kb band. 26 of the 33 stable expressers produced sectors with no visible GFP activity during the culture period. Southern analysis of DNA from 7 of these cell lines was unable to detect *gfp* sequences in 2 and detected less than one copy per genome in 1. The remaining 4 cell lines showed no evidence of T-DNA loss and 1 had the high copy number 5-7kb band (Figure 4. 3. 8). 3 cell lines stably expressed GFP throughout the experiments without producing any observable sectors lacking visible GFP activity. *gfp* sequences were detected by Southern analysis in all 3 cell lines and none had the high copy number band.

4. 3. 4. Gene silencing

In the 10 transient expressers that retained T-DNA sequences, loss of GFP activity could be due to gene silencing. However, it is also possible that only mutated or partially deleted copies of the *gfp* expression cassette were retained in the host genome and that functional *gfp* genes responsible for transient expression were lost from the cells. This latter explanation seems unlikely given that 9 of the 10 cell lines contained many copies of T-DNA. The 10 transient expressers that retained T-DNA sequences were monitored for GFP activity weekly over a two-month period. During the two months, 4 cell lines produced cells with visible GFP activity suggesting that these cell lines contained silenced, intact *gfp* sequences. Thus, if mutation is the mechanism behind the apparent silencing, over time it must revert or be repaired by recombination. In the latter case, repair requires two templates with different mutations. Whereas this remains a formal possibility, other mechanisms of gene silencing with less complex requirements could explain the results.

To further test for evidence of gene silencing, the 10 cell lines were sub-cultured onto medium containing 0.5mM 5-azacytidine, an agent that inhibits the maintenance of cytosine methylation (Figure 4. 3. 9). Methylation has been implicated in epigenetic control of gene expression. After 3 days growth on this medium, the cells were transferred to medium lacking 5-azacytidine. Within a week of transfer, all cell lines produced one or two small sectors of GFP activity. These sectors typically involved only a few cells, representing only a small percentage of the cells plated on 5-azacytidine. GFP activity faded over a period of a few days.

This initial experiment was repeated in three further experiments with a range of 5-azacytidine concentrations (0.2mM, 0.5mM, 0.8mM, 1.0mM and 5mM). In these experiments, the cells were maintained on the medium containing 5-azacytidine for at least 14 days. Six cell lines regularly produced high numbers of cells with strong GFP expression. 3 cell lines produced cells strongly expressing GFP in one or two treatments and 1 cell line did not produce any cells with visible GFP in any of these 3 experiments. Gene silencing was probably a cause of loss of *gfp* expression in at least 9 of the 10 transient expressers that retained T-DNA sequences.

If the treatment with 5-azacytidine caused the observed GFP activity by demethylating silenced loci, then it should be possible to detect methylated *gfp* sequences within the non-expressing cell lines. To test for methylation, genomic DNA from five cell lines lacking GFP activity and DNA from two cell lines with GFP activity were digested with *Sau*3AI and PCR was used to test for the presence of undigested *gfp* sequences. There are 4 *Sau*3AI restriction sites within the *gfp* gene. Cleavage by *Sau*3AI is inhibited by cytosine methylation. If all four restriction sites were methylated, digestion with *Sau*3A1 would leave the *gfp* gene intact and PCR amplification of the *gfp* gene would be possible. If any of the *Sau*3AI restriction sites were unmethylated the *gfp* gene would be cut by *Sau*3AI and PCR amplification would not be possible. The PCR results demonstrate that amplification of *gfp* sequences from the two cell lines that contained GFP activity, after digestion with *Sau*3AI, requires 10-500 fold more DNA than amplification of *gfp* sequences from the 5 cell lines containing silenced *gfp* sequences (Figure 4. 3. 10). While this result might indicate that *gfp* sequences are more highly methylated in cell lines that do not express those sequences, it is also possible that the difference in PCR amplification

is due to differences in the DNA samples (eg, *gfp* copy number, DNA purity and concentration). As a control for variation between DNA samples, a dilution series of undigested DNA from each DNA sample was also tested for the presence of *gfp* sequences by PCR amplification (Figure 4. 3. 11). While there were differences between the cell lines in the quantity of DNA required for PCR amplification of the *gfp* sequence, these differences were not sufficient to account for the differences in PCR amplification after Sau3AI digestion.

As a further test for cytosine methylation, DNA from 4 transiently expressing cell lines was digested with either *Sau*3A1 or *Nde*II. *Sau*3A1 and *Nde*II both cleave DNA at the sequence GATC. However, while cleavage by *Sau*3A is inhibited by cytosine methylation, cleavage by *Nde*II is not. If all four GATC sites within the *gfp* gene are methylated, digestion with *Sau*3A1 will leave the *gfp* gene intact while *Nde*II digestion should leave no *gfp* sequences intact. After digestion, PCR amplification from primers flanking the *gfp* gene was used to detect undigested *gfp* sequences. The results suggest that *Sau*3AI digestion was inhibited but that *Nde*II digestion was not inhibited in 4 of the 5 cell lines tested. This result suggests that the 4 cell lines contained methylated *gfp* sequences (Figure 4. 3. 12 lanes 1, 2, 4, 5). The differences in PCR amplification after restriction digestion was unlikely to be due to *Sau*3AI being less active than *Nde*II as *Nde*II was approximately 30 fold less active than *Sau*3A1 when cutting unmethylated plasmid DNA (Figure 4. 3. 13). However, there were some unexpected results in this experiment. Some PCR amplification product was observed after *Nde*II digestion (eg. Figure 4. 3. 12, lane 17). This result was consistently observed and possibly reflects the unexpectedly low activity of the *Nde*II enzyme used. DNA methylation would only be indicated if there was consistently a PCR product from Sau3A-digested DNA and no PCR product from NdeII—digested DNA. Also, PCR consistently did not amplify undigested DNA taken from one cell line (Figure 4. 3. 12, lane 11). Further, a faint band was occasionally seen in one negative control (DNA from an untransformed cell line) (Figure 4. 3. 12, lane 20). While these anomalies make this particular experiment unconvincing they do not detract from the previously described experiment that indicated that GFP gene sequences in the transient expressing cells were methylated.

Some causes of gene silencing (eg. T-DNA integration into heterochromatin and pairing of homologous loci) would stop expression from all genes on the T-DNA. Some stable and all transient expressers were tested for loss of expression of the kanamycin resistance gene (*npt*II) which was also carried on the T-DNA of *pBINmgfp5-ER*. The expression of the *nptII* gene was examined by culturing cells on CS-V medium supplemented with kanamycin at a range of concentrations (0mg/l, 50mg/l, 100mg/l, 200mg/l, 300mg/l, 400mg/l, 500mg/l) (Figure 4. 3. 14). Of the 10 transient expressers that retained *gfp* sequences, only 1 was resistant to kanamycin. Of the 6 stable *gfp* expressers tested, 5 were resistant to kanamycin. This result suggests that, in some instances, the *gfp* gene was silenced by a mechanism that effects the T-DNA loci generally.

4. 3. 5. Growth rate of calli

During experiment 1, calli that had lost GFP activity appeared to grow more slowly than those that retained GFP expression. To investigate this further, the diameter of calli four weeks after T-DNA transfer was recorded in experiments 2 and 3. In both of these experiments, the average diameter of transiently expressing calli was smaller than both the diameter of stably expressing calli and calli derived from protoplasts that never expressed GFP (Table 4. 3. 2). There was no difference in diameter between proto-calli stably expressing GFP and calli that had never expressed GFP. In an analysis (ANOVA) of data pooled from both experiments, the average diameter of transiently expressing calli was significantly (at the 5% level) less than the average diameter of protoplasts that had never expressed GFP. The difference between the average diameter of stably and transiently expressing cell lines, while consistently high, was not significant at the 5% level. These results suggest that the transiently expressing cell lines have an attenuated growth rate.

Table 4. 3. 2: Diameter of *N. plumbaginifolia* calli four weeks after co-cultivation. Pooled data from experiments 2 and 3.

	GFP+[1]	GFP-[2]	Untransformed[3]
Average diameter (mm)	0.635	0.444	0.700

Mean LSD 5%=, 0.2103, df= 65

1: GFP+ = calli with GFP activity four weeks after co-cultivation.

2: GFP- = calli with GFP activity four days after co-cultivation but with no activity at four weeks.

3: Untransformed = calli with no GFP activity at four days and at four weeks after co-cultivation.

The least significant difference (LSD) is derived from an analysis of variance. Because the three samples contain different numbers of replicates, a mean LSD at 5% is given. There is no difference between GFP+ and untransformed cell lines. There is a significant difference at the 5% level between GFP- and untransformed cell lines. The difference between GFP- and GFP+ cell lines is not significant at the 5% level.

The time of first division of a sample of marked protoplasts was also recorded. There was no correlation between the time of first division and either transient expression or calli size. However, amongst protoplasts that transiently expressed GFP, those that divided relatively soon after co-cultivation more often contained transgenic loci than late dividers (Figure 4. 3. 15). Only the 9 transient expressers recovered from experiment 2 were included in this analysis. Not enough data from experiment 3 was obtained for analysis. These results suggest that there may be two distinct phenomena: the relationship between

time of first division and T-DNA integration; and the relationship between growth rate and transient expression.

Figure 4. 3. 1.
N. plumbaginifolia protoplasts after 5 hours digestion with cell wall degrading enzymes.

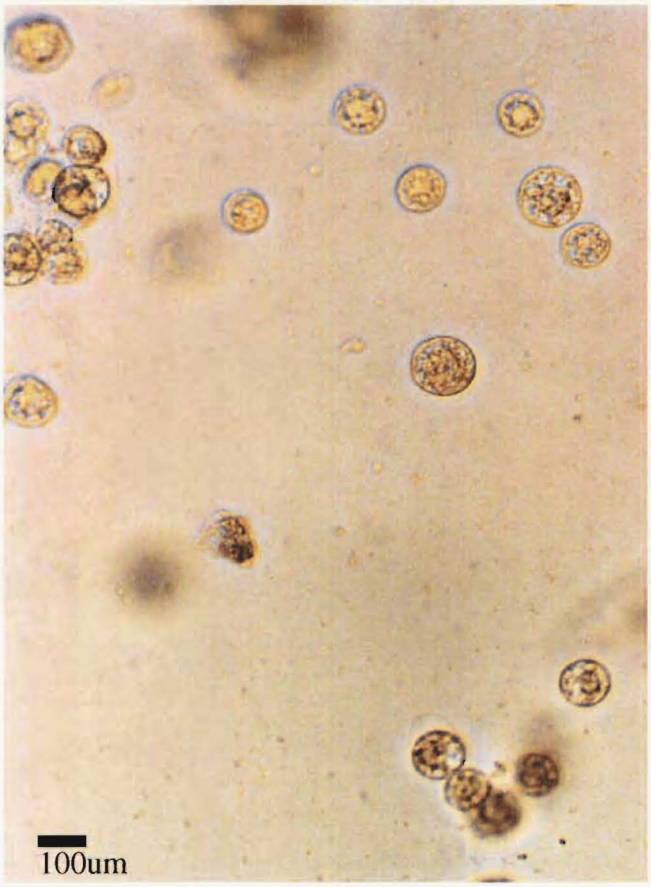


Image illuminated with white light.

Figure 4. 3. 2.
N. plumbaginifolia protoplasts embedded in 0.4M CS-V medium solidified with 1% agarose.

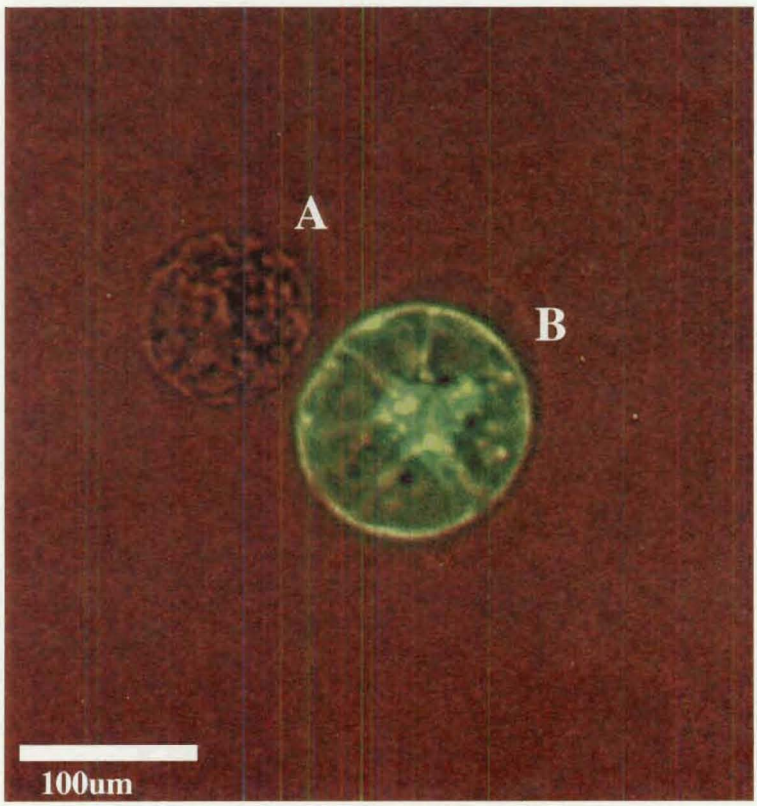


Image illuminated with blue and white light.

A: *N. plumbaginifolia* protoplast without visible GFP activity.

B: *N. plumbaginifolia* protoplast with visible GFP activity.

Figure 4. 3. 3.
N. plumbaginifolia protoplast first division after 14 days culture embedded in 0.4M CS-V medium, 1% agarose.

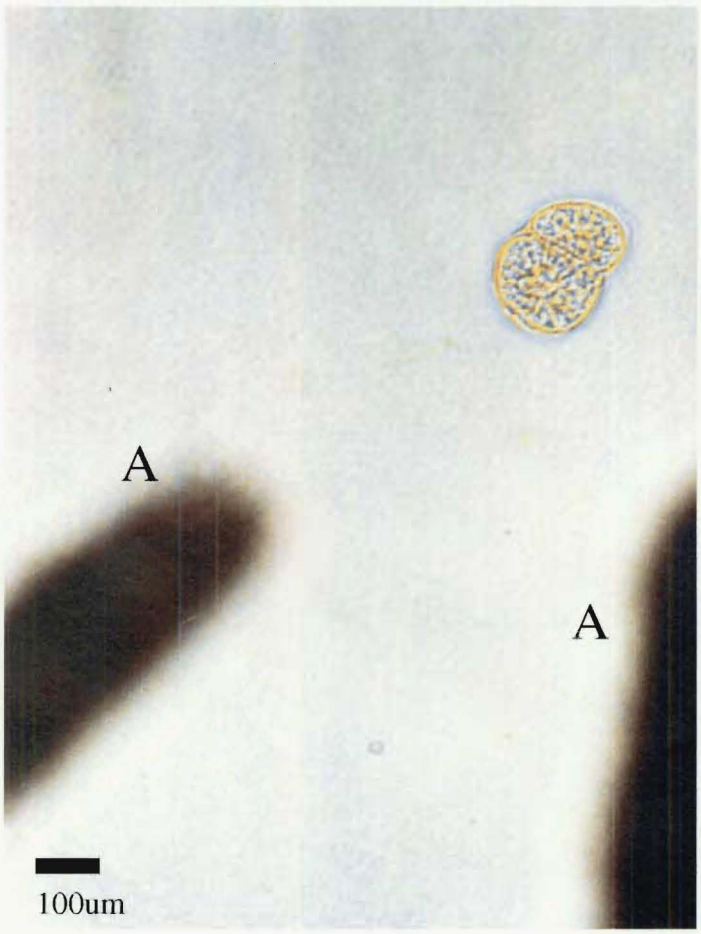


Image illuminated with white light.

A: Locating marks scratched on surface of petri dish.

Figure 4. 3. 4.
N. plumbaginifolia proto-callus after 14 days culture embedded in 0.4M CS-V medium, 1% agarose.

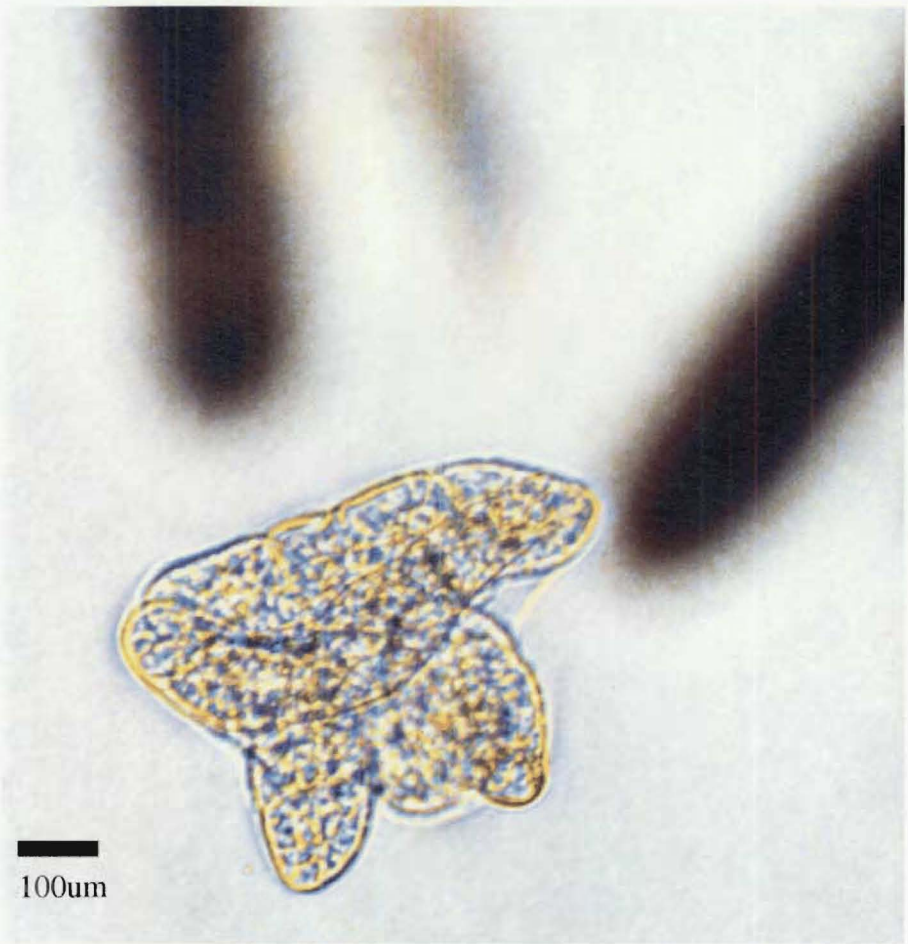


Image illuminated with white light.

Figure 4. 3. 5.
N. plumbaginifolia callus with stable GFP activity embedded in 0.4M CS-V medium, 1% agarose after 4 weeks growth.

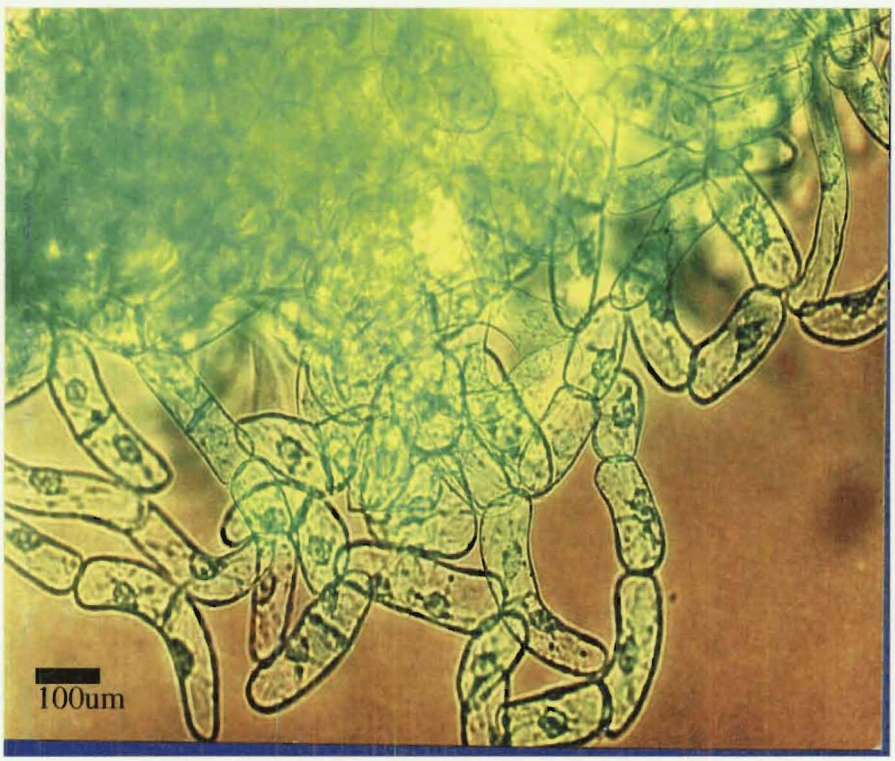
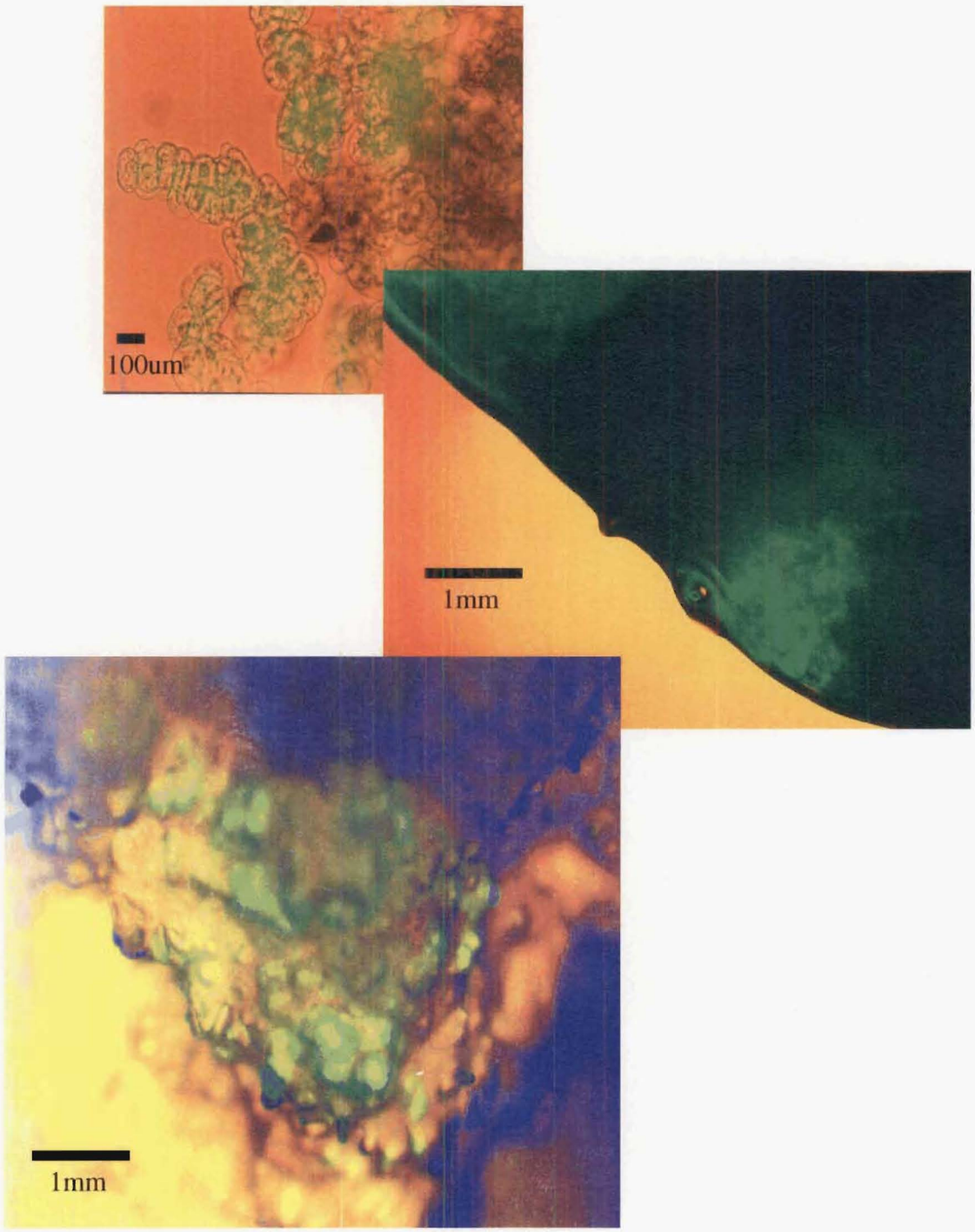


Image illuminated with blue light and white light.

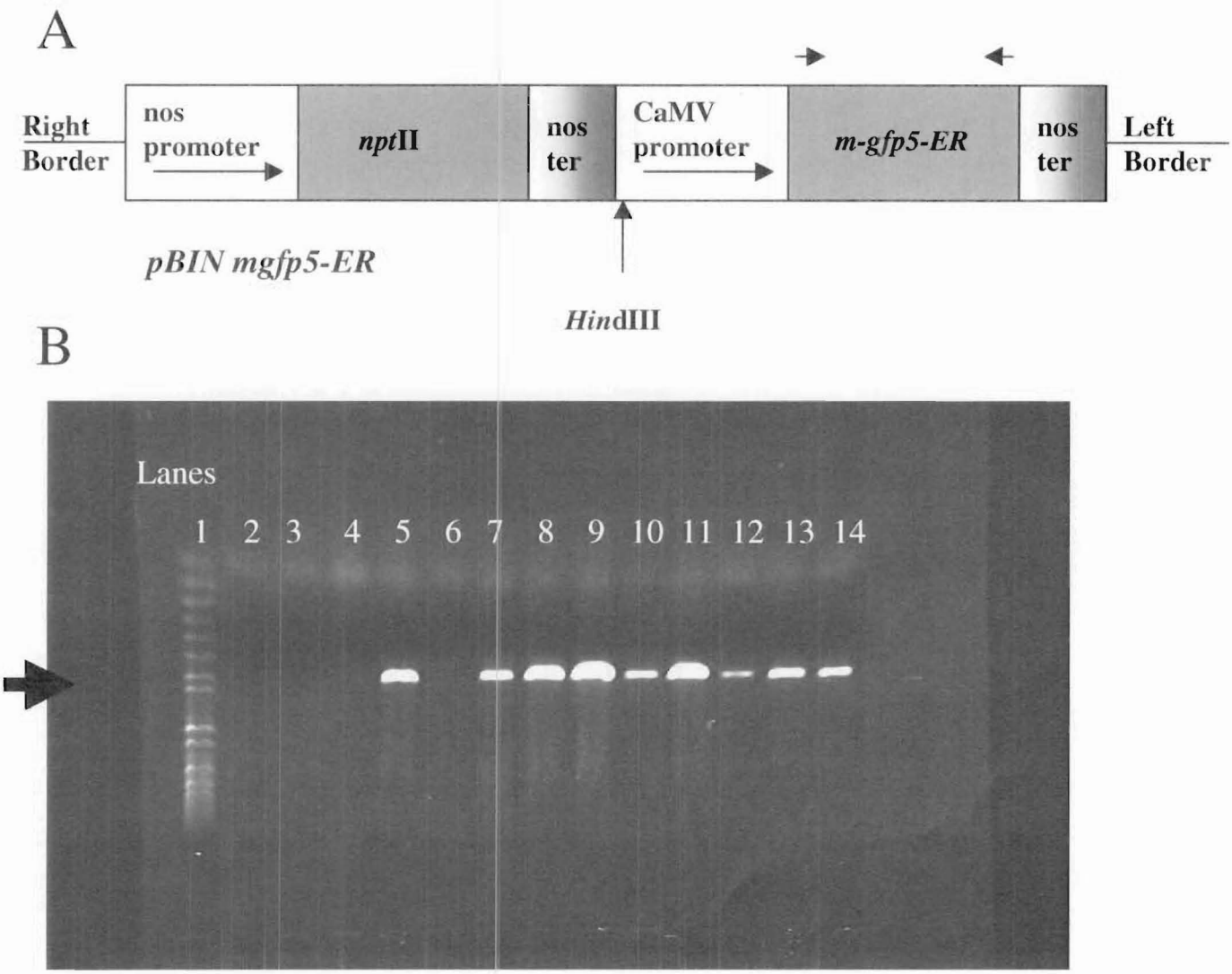
Figure 4. 3. 6.
N. plumbaginifolia calli derived from individual protoplasts showing sectoring for visible GFP activity.



Images illuminated with blue light and white light. Green areas are expressing GFP.

Figure 4. 3. 7. 146

PCR of *N. plumbaginifolia* cell lines with primers specific to the *m-gfp5-ER* gene.



A: Map of plasmid *pBIN m-gfp5-ER*.

→ ← PCR primer sites used to amplify *m-gfp5-ER* gene.

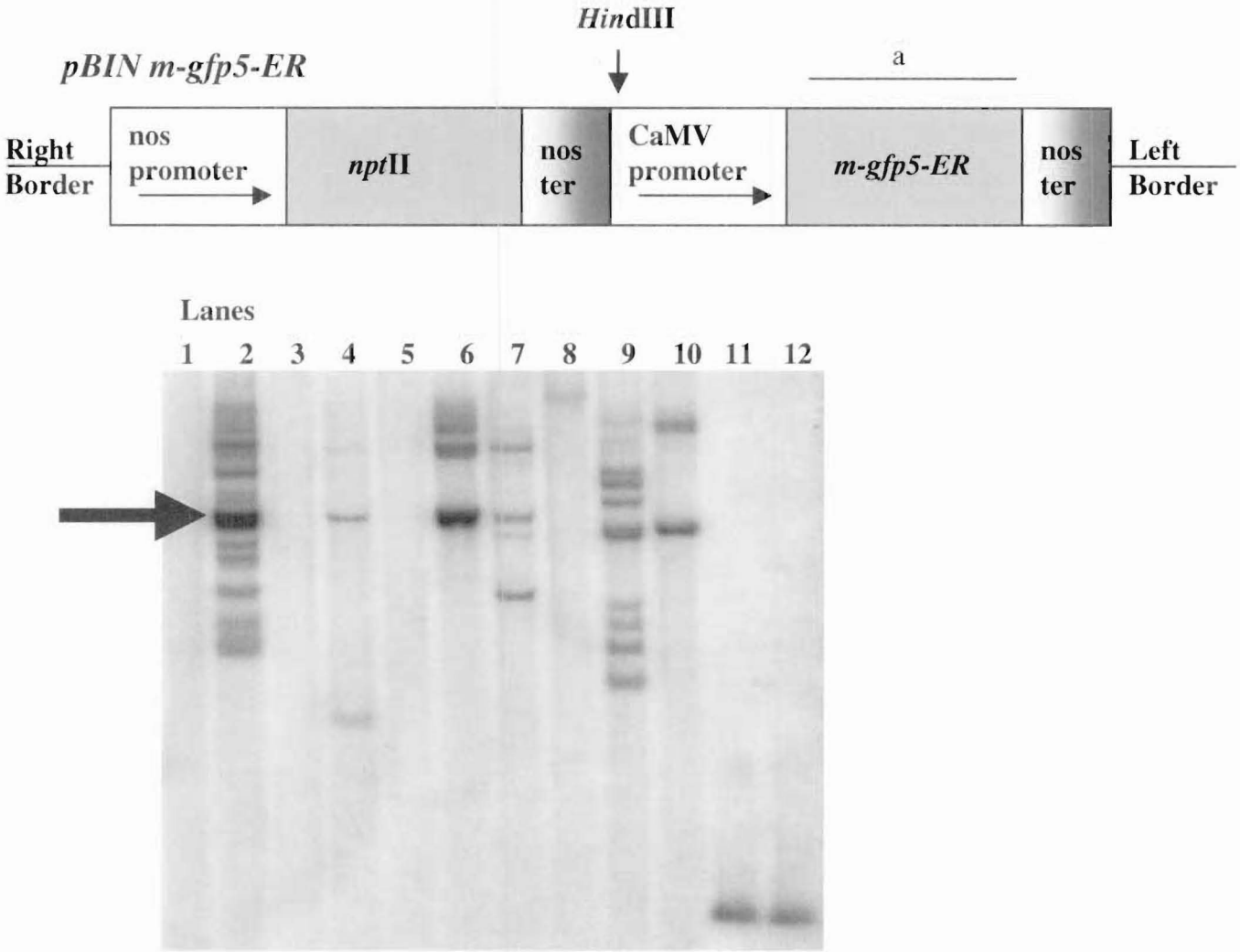
B: Product of PCR using *gfp*-specific primers. Lane 1: 1kb ladder; lane 2: water control; lanes 3 and 4: untransformed suspension cells; lanes 5-10: transiently expressing cell lines; lanes 11-14: stably expressing cell lines.

➡ Expected 820bp PCR amplification product.

*npt*II: kanamycin resistance gene, *m-gfp5-ER*: GFP gene, *Hin*dIII: recognition site for restriction enzyme *Hin*dIII. Right and left borders: Right and left T-DNA border sequences. CaMV promoter: califlower mosaic virus 35S promoter, nos ter: transcription terminator sequence from nopaline Ti plasmid. nos promoter:promoter from nopaline Ti plasmid.

Figure 4. 3. 8.

Autoradiograph of *Hin*dIII digested *N. plumbaginifolia* genomic DNA hybridised to a probe specific to the *gfp* gene.



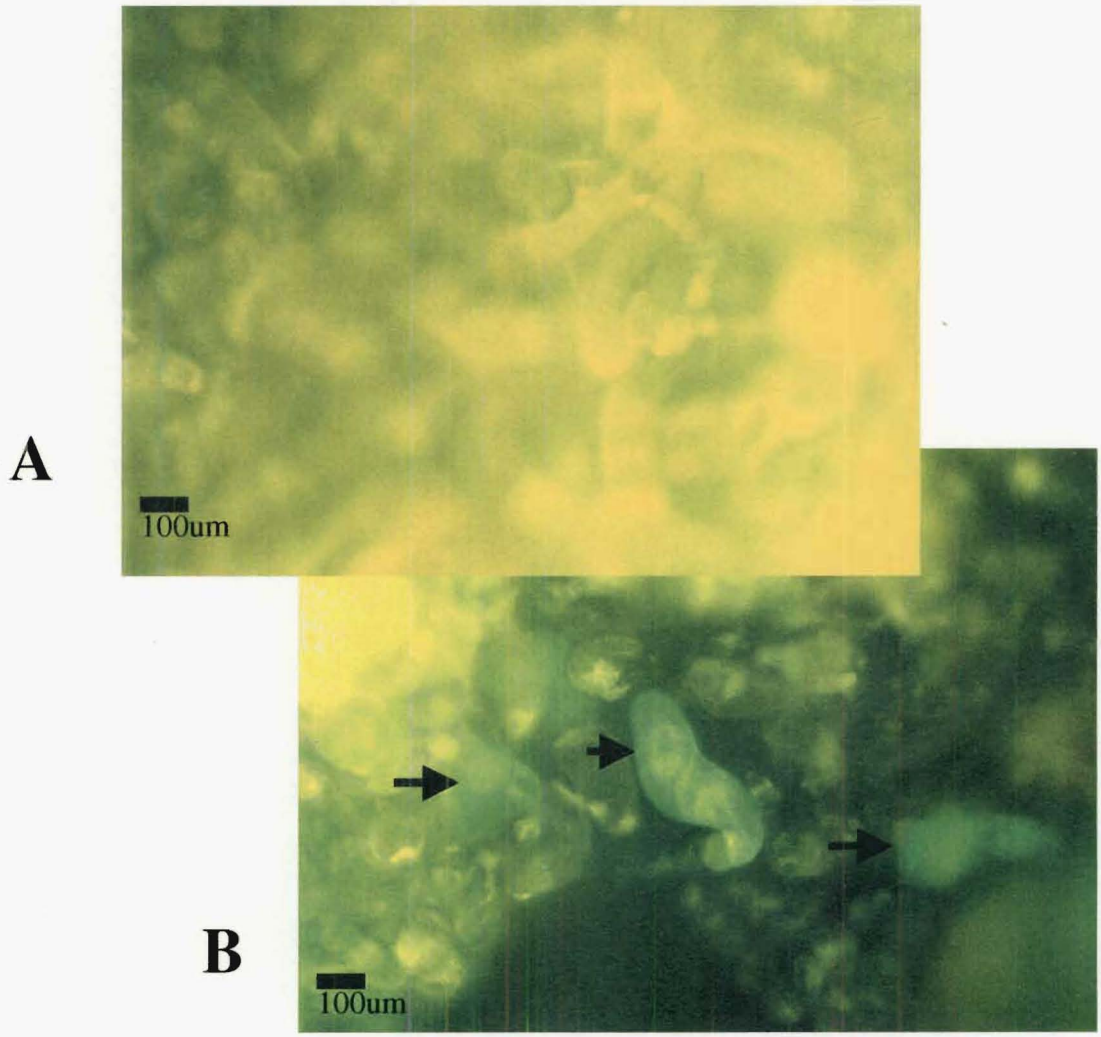
Lane 1: untransformed cell line; lanes 2-6: transient expressers; lanes 7-10: stable expressers; lanes 11 and 12: T-DNA copy number controls (2 and 1 copy per genome, respectively).

→ position of high copy number band of approximately 5kb.

*npt*II: kanamycin resistance gene, *m-gfp5-ER*: GFP gene, *Hin*dIII: recognition site for restriction enzyme *Hin*dIII. Right and left borders: Right and left T-DNA border sequences. CaMV promoter: califlower mosaic virus 35S promoter, nos ter: transcription terminator sequence from nopaline Ti plasmid. nos promoter: promoter from nopaline Ti plasmid.

a: P^{32} dCTP-labelled probe homologous to the *m-gfp5-ER* gene

Figure 4. 3. 9.
N. plumbaginifolia cell line treated with 5-azacytidine resulting in restoration of GFP activity.

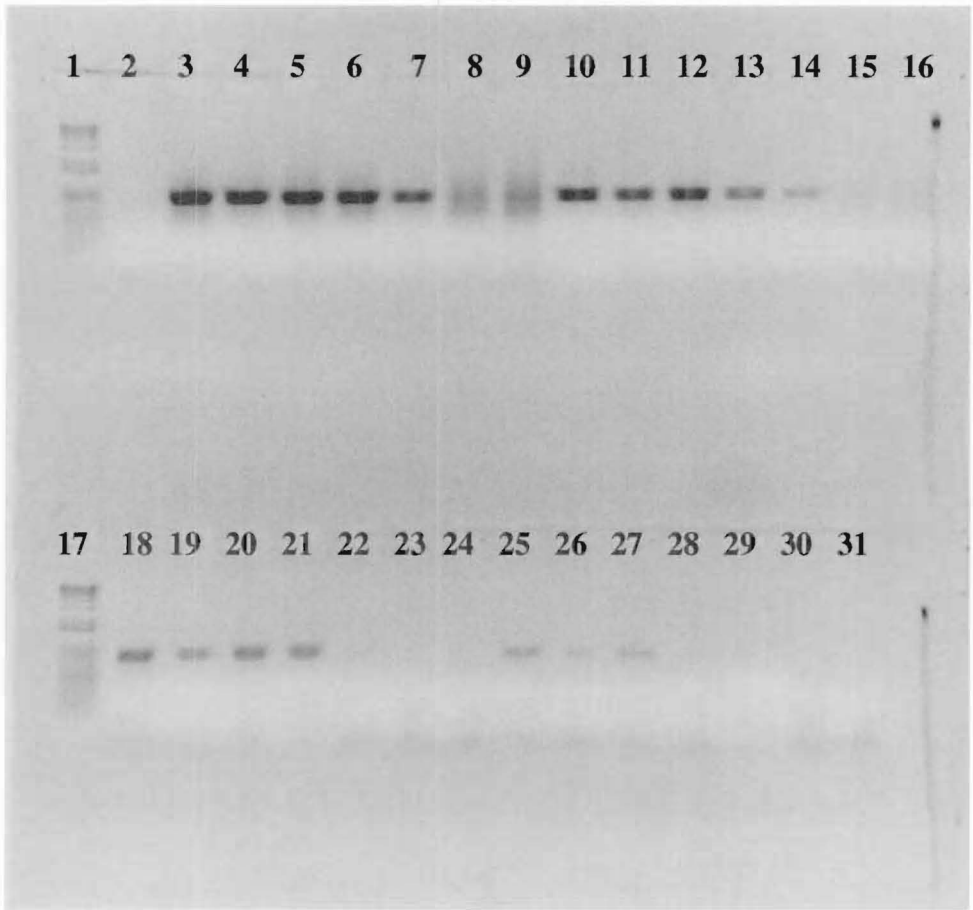


A: Transiently expressing cell line prior to treatment.

B: The same cell line two weeks after treatment with 5-azacytidine.

➔ Cells with GFP activity.

Figure 4. 3. 10.
PCR amplification of the *gfp* gene from *N. plumbaginifolia* genomic DNA digested with *Sau*3A.



Lanes 1 and 17: 1kb ladder. Lane 2: water control.

Lanes 3-9: PCR product from amplification of 2ul *Sau*3A digested genomic DNA from NA2, OE13, OA32, NZ, OB23, OB24 (stable GFP), and OA16 (stable GFP), respectively.

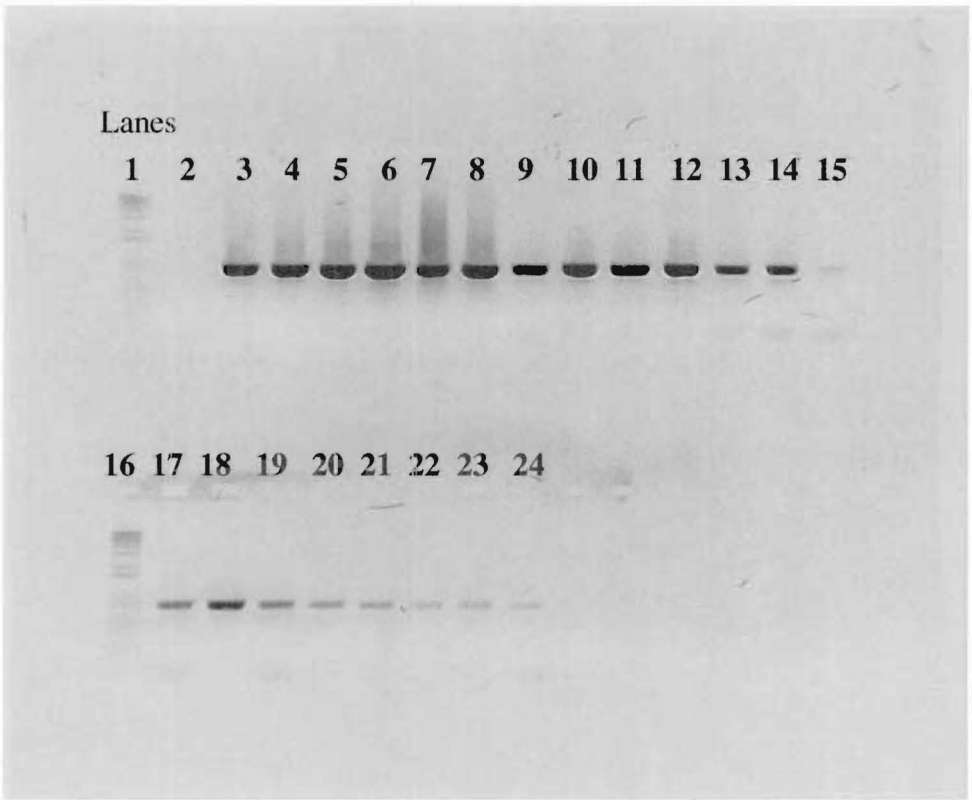
Lanes 10-16: PCR product from amplification of 1/5ul *Sau*3A digested genomic DNA from NA2, OE13, OA32, NZ, OB23, OB24 (stable GFP), and OA16 (stable GFP), respectively.

Lanes 18-24: PCR product from amplification of 1/50ul *Sau*3A digested genomic DNA from NA2, OE13, OA32, NZ, OB23, OB24 (stable GFP), and OA16 (stable GFP), respectively.

Lanes 25-31: PCR product from amplification of 1/250ul *Sau*3A digested genomic DNA from NA2, OE13, OA32, NZ, OB23, OB24 (stable GFP), and OA16 (stable GFP), respectively.

The PCR assay to detect *gfp* sequences in the stable expressers OB24 and OA16, after *Sau*3A digestion, requires 10 fold more template DNA than the assay to detect *gfp* sequences in the transient expresser OB23; 100 fold more DNA than the assay to detect *gfp* sequences in the transient expresser NZ; and 500 fold more DNA than the assay to detect *gfp* sequences in NA2, OE13 and OA32.

Figure 4. 3. 11.
PCR amplification of the *gfp* gene from undigested *N. plumbaginifolia* genomic DNA.



Lanes 1 and 16: 1kb ladder. Lane 2: water control.

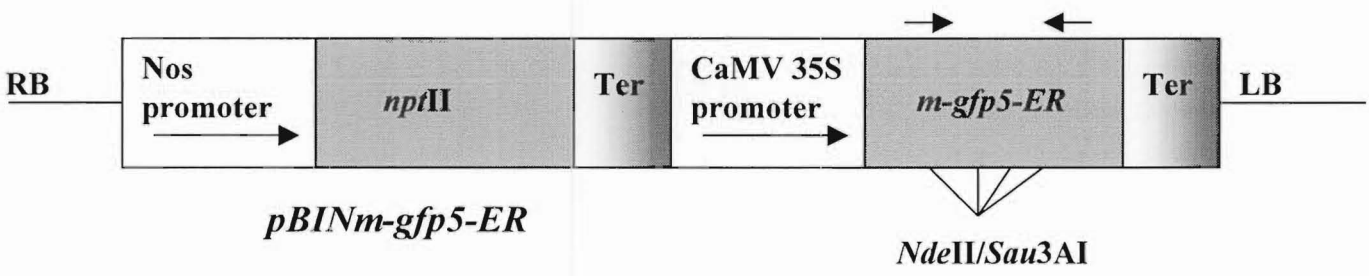
Lanes 3-8: PCR product from amplification of 2ul genomic DNA from NZ, OE13, NA2, OA32, OA16 (stable GFP), OB24 (stable GFP), respectively.

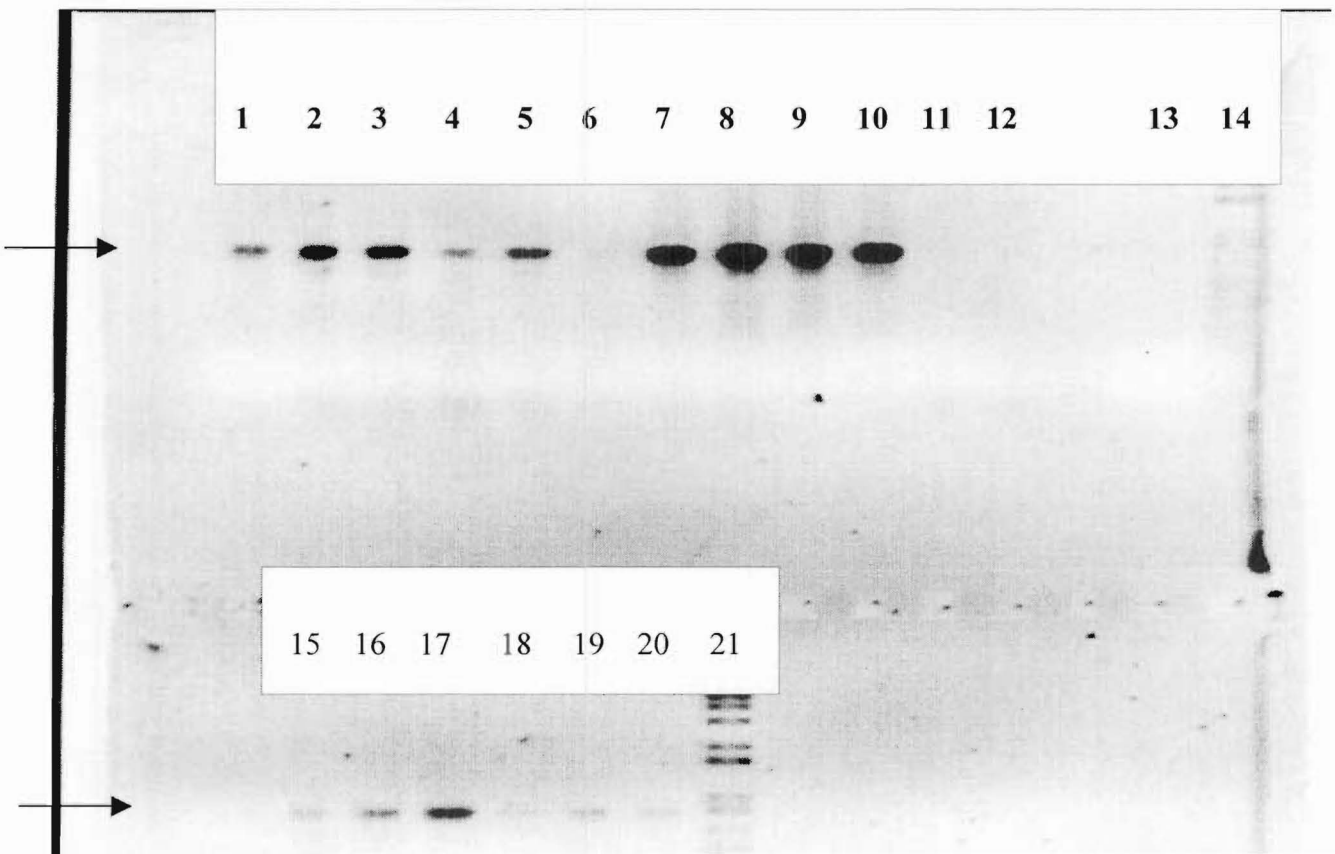
Lanes 9-14: PCR amplification product from 1/10ul genomic DNA from NZ, OE13, NA2, OA32, OA16, OB24, respectively.
from
Lane 15 and lanes 17-21: PCR product amplification of1/100ul genomic DNA from NZ, OE13, NA2, OA32, OA16, OB24, respectively.

Lanes 22-24: PCR product from amplification of 1/1000ul genomic DNA from OE13, NA2 and OA32 respectively.

The sensitivities of the PCR assays to detect *gfp* sequences in undigested DNA from the stable GFP expressers OA16, OB24 and the transient expresser NZ are essentially the same. The assays to detect *gfp* sequences in undigested DNA from the transient expressers OE13, NA2, and OA32 are approximately 10 fold more sensitive.

Figure 4. 3. 12.
PCR of *N. plumbaginifolia* cell lines with primers specific to the *m-gfp5-ER* gene after digestion with either *Nde*II or *Sau*3A.

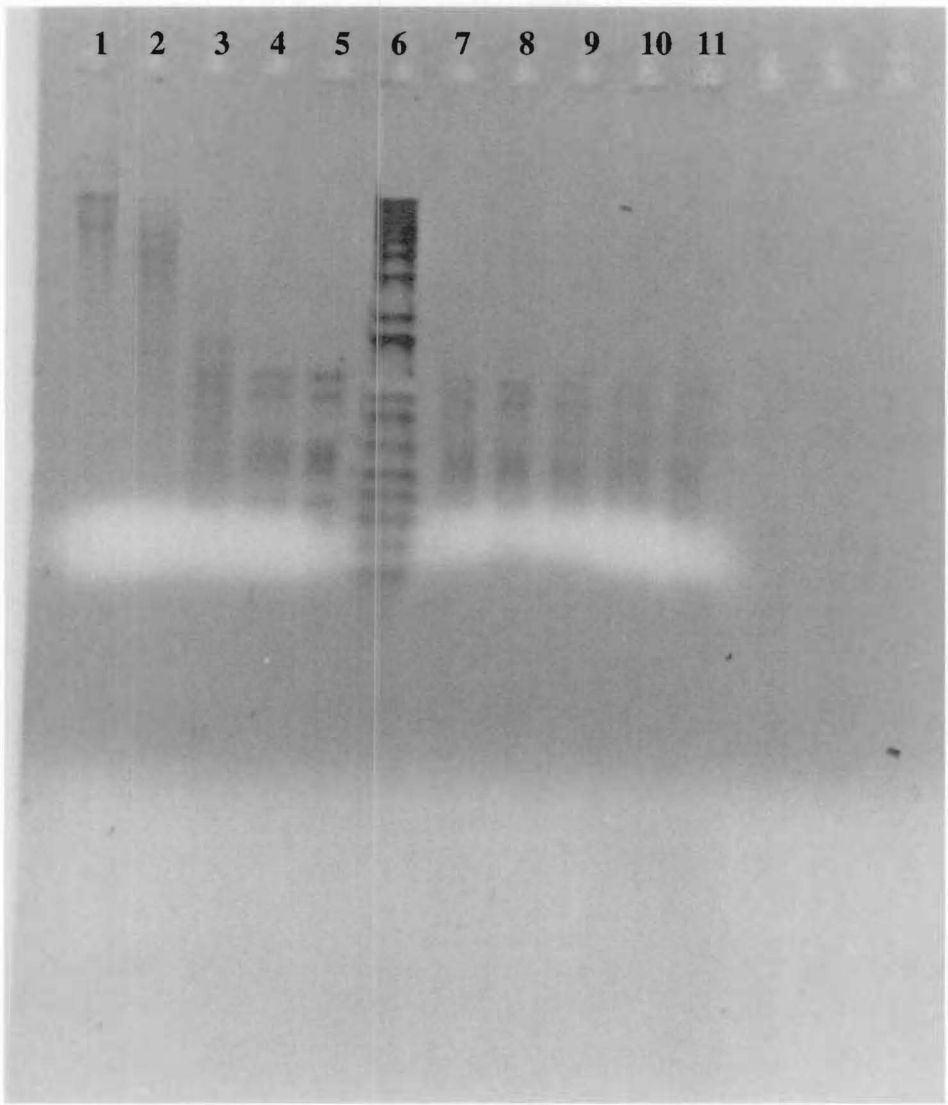




Lanes 1-6: PCR amplification product after genomic DNA digested with *Sau*3A(DNA from transient expressers NZ-, NA2, OE13, NZ+, OB23 and untransformed cell line ODG respectively. Lanes 7-12: PCR amplification product after genomic DNA incubated without digestion (DNA in same order as lanes 1-6). Lane 13: water control. Lanes 15-20: PCR amplification product after genomic DNA digested with *Nde*II (DNA in same order as lanes 1-6). Lanes 14 and 21: 1kb ladder. Arrows show position of expected 820bp *gfp* PCR product.

→◄← PCR primers, *npt*II: encodes kanamycin resistance, *m-gfp5-ER*: encodes GFP, *Sau*3AI: cleavage site for restriction enzyme *Sau*3AI, *Nde*II: cleavage site for restriction enzyme *Nde*II, Ter: transcription terminator sequence. RB: right T-DNA border sequence; LB: left T-DNA border.

Figure 4. 3. 13.
Restriction digest of unmethylated plasmid DNA with *Sau*3A and *Nde*II.

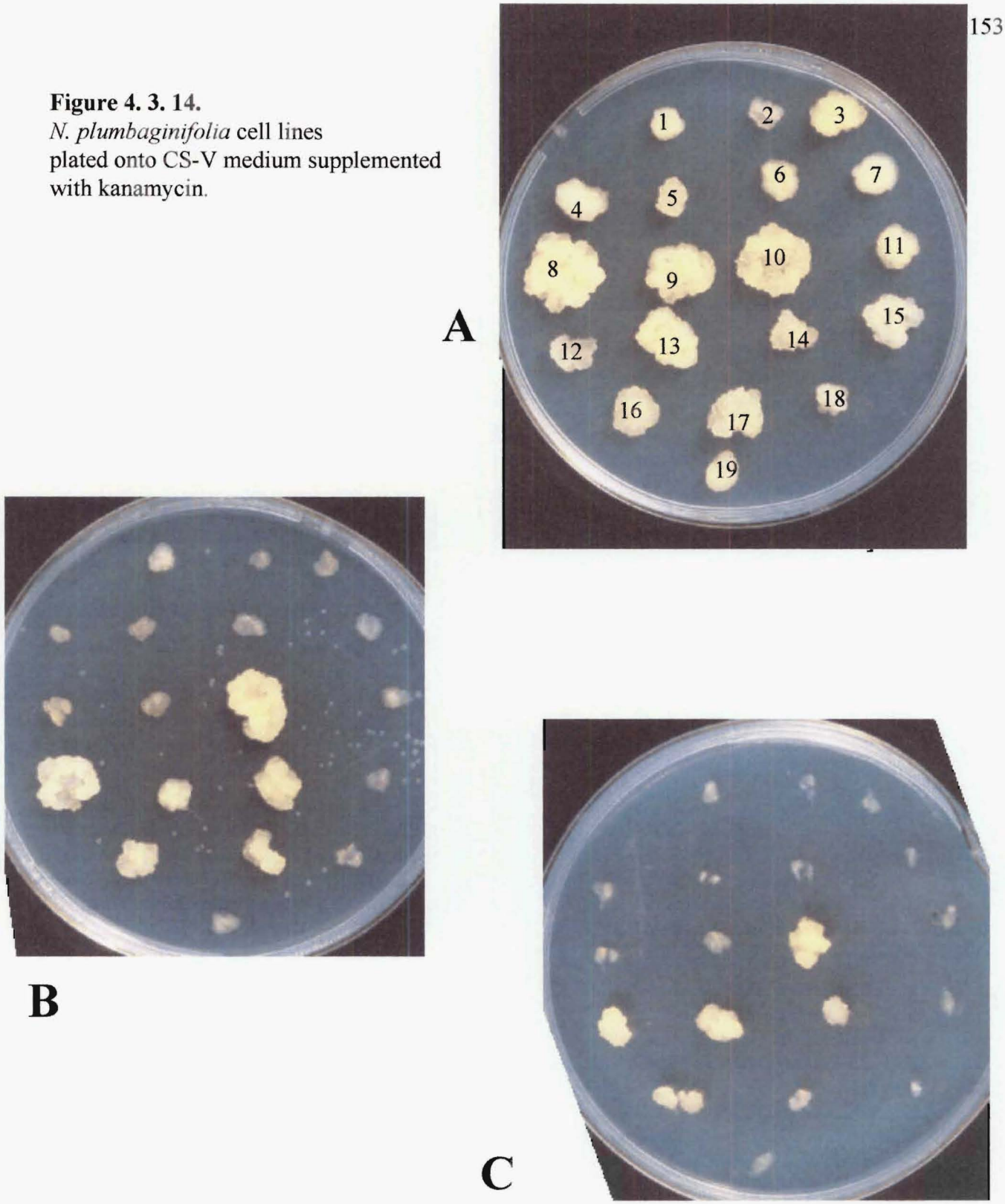


Lanes 1-5: *Nde*II digested DNA. In each consecutive lane (from lane 1 to lane 5) a three fold higher concentration of *Nde*II was used in the DNA digestion.

Lane 6: 1kb ladder.

Lanes 7-11: *Sau*3A digested DNA. In each consecutive lane (from lane 7 to lane 11) a three fold higher concentration of Sau3A was used in the DNA digestion. The same concentration of restriction enzyme was used in lanes 1 and 7 etc.

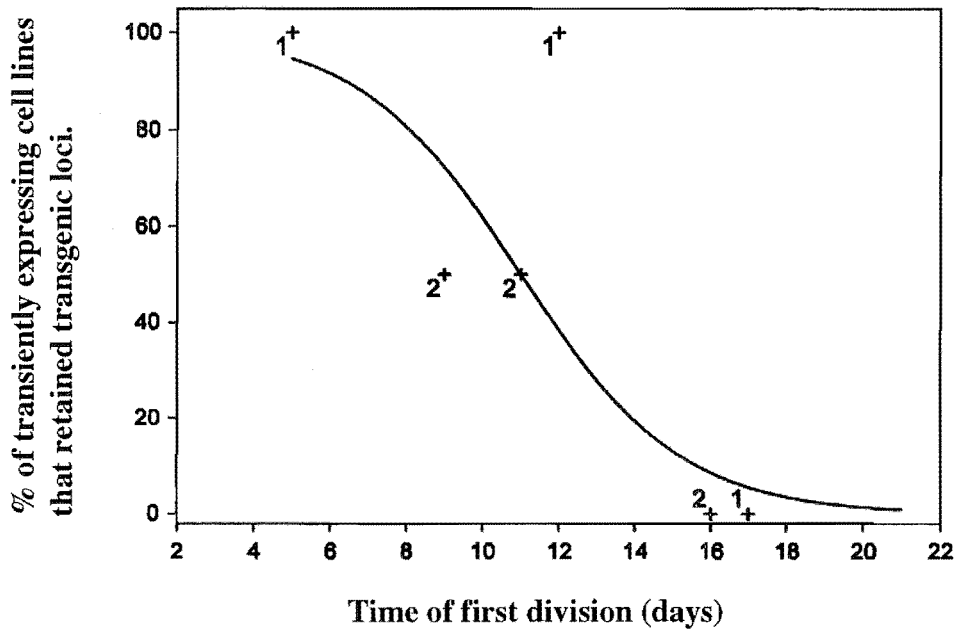
Figure 4. 3. 14.
N. plumbaginifolia cell lines plated onto CS-V medium supplemented with kanamycin.



A: 0mg/l kanamycin, B: 100mg/l kanamycin, C: 300mg/l kanamycin. Cell lines are in the same order on each plate. Transient GFP expressers are numbers: 1, 2, 4-10, 15. Stable GFP expressers are numbers: 12-14, 16-18. Untransformed cell lines are numbers: 1, 11, 19.

Some kanamycin resistance is evident in 5 stable expressers (numbers: 12, 13, 14, 16, 17) and 1 transient expresser (number 10).

Figure 4. 3. 15.
Relationship between the time of first division of *N. plumbaginifolia* protoplasts that transiently expressed GFP and the presence of T-DNA loci in the resulting cell lines.



Parameter	Estimate	s.e. (df=7)
slope	0.476	0.429
T50	11.01	2.09

The percentage of cell lines showing transgenic loci for each time of first division was modelled with a logistic curve, with 100% of cell lines retaining transgenic loci at the maximum, declining to 0% of cell lines retaining transgenic loci:

$$\% \text{ with loci} = 100 / 1 + e^{\text{slope} * (\text{time-T50})}$$

Where: slope is related to the steepness of the curve at time = T50,
and T50 is the time where only half the cell lines produced transgenic loci.

The total number of cell lines at each time of first division is shown adjacent to the data.

4. 4. Discussion

The transient nature of GFP expression in this system is primarily associated with, and presumably caused by, cell death. The extent to which cell death affects stable transformation rates following *A. tumefaciens*-mediated gene transfer is unknown and not widely considered in the literature. One study of microprojectile bombarded tobacco leaf explant found that 90% of transiently expressing cells were dead 2 days after bombardment (11). Cell death could account for all transient expression events observed in that study. In *A. tumefaciens*-mediated gene transfer, cell death may result specifically from T-DNA transfer, either directly from genomic integration or from genetic lesions caused by error prone repair of integration intermediates (20). However, we found no relationship between T-DNA transfer (as measured by GFP activity) and cell mortality. The high mortality rate in our study was probably primarily due to natural cell turnover and the harshness of the culture conditions required to isolate individual transformed cells. Cell mortality, in our experiments, was similar to mortality rates observed by other researchers using similar plant culture systems (18).

Contrary to expectations, we found that most recovered transient expressers (10/17) contained multiple T-DNA inserts. This result contradicts previous studies, which suggested that transient expression was primarily associated with loss of T-DNA sequences (3, 41). In one study, De Buck et al. (3) transferred two T-DNA constructs (carrying different selectable marker genes) into tobacco cells by co-cultivation with *A. tumefaciens*. After co-cultivation, cell lines were recovered without selection and then screened for expression of the two constructs. Clones that expressed only one of the two marker genes were then tested for the presence of the unexpressed construct by hybridisation to a labelled probe homologous to the transferred DNA. A sample of clones that did not express either marker was also examined. Transformants with a silenced marker gene were very rare (3). However, as this study shows, transient expressors may grow more slowly than other untransformed cells. Also, the method used to enrich for transient expressers may have left unsampled those cells that simultaneously silenced both genes. Therefore, it is possible that De Buck et al. did not obtain a representative sample of transient expressers. It is also possible that our *N. pulmbaginifolia* cell suspensions, and the *N. tabacum* leaf protoplasts used by De Buck's group, reacted differently to T-DNA transfer. In particular, leaf mesophyll cells are at G0-G1 in the cell cycle whereas our

suspension cells were unsynchronized at the time of co-cultivation. The phase in the cell cycle probably has an important role in T-DNA stabilisation.

In a further study, Vergunst and Hooykaas (41) concluded that the transient nature of T-DNA expression was not due to the absence of cell proliferation or gene silencing, but to loss of gene copies. In an experiment where T-DNA carrying the *cre* gene and T-DNA carrying *lox* sites were transferred into *A. thaliana* plant cells, they isolated *lox* recombinants lacking the *cre* vector. As *cre* expression was probably required for *lox* recombination, *cre* was most likely transiently expressed and subsequently lost from the plant cells. However, 5 of the 15 lox recombinants retained *cre* sequences. Furthermore, the regenerants were probably not a representative sample of transient expressers as their sampling method required more than just loss of T-DNA expression. Even in the very direct sampling approach undertaken here, a random sample of transient expressers was not obtained as some of the slowest growing calli were lost. With this exception, a representative sample of all transient expressers was obtained. I propose that gene silencing and cell death are at least as significant causes of loss of expression as is total loss of T-DNA.

It is now known that the expression of transgenes integrated into plant chromosomes is not constant but is subject to epigenetic regulation (reviewed in 21 and 40). Most studies of transgene silencing involved changes in expression in regenerated plants (12, 31, 37, 45). Little is known about transgene silencing events that occur immediately after gene transfer. However, we do know that gene transfer can quickly lead to co-suppression of homologous genes that are stably integrated into the plant genome (2, 35, 43). Co-suppression of a chromosomally integrated *gfp* transgene has been shown to occur within 8 days of inoculation with plasmids containing the *gfp* gene (35). Also, silencing of a chromosomal *gfp* gene in tobacco had occurred by 2 days after transfer of T-DNA carrying a promoterless *gfp* gene to the tobacco leaf cells (43). Our results indicate that some expression is routinely lost through gene silencing. That gene silencing provides a substantial barrier to stable transformation has previously been suggested by the observation that co-cultivation of tobacco cells with an *A. tumefaciens* strain carrying a mutation in the Ti plasmid *vir*C resulted in a relatively low frequency of T-DNA transfer (as judged by transient T-DNA expression) but a high frequency of stable transformation

(24). The high stable transformation frequencies obtained using a transfer defective *A. tumefaciens* strain is consistent with the observation that gene silencing is frequently associated with multiple transgene integrations (1, 4, 31, 45). Also, the efficiency of transformation of *Arabidopsis thaliana* with T-DNA has been increased by treating regenerating cells with the C-methylation inhibitor 5-azacytidine (19). This result suggested a role for gene silencing in transient expression as C-methylation of promoter sequences has been implicated in gene inactivation (32).

A significant proportion of recovered transient expressers (7/17) lacked *gfp* sequences. It is not certain whether transient expression in these cases occurred from extrachromosomal T-DNA or from T-DNA integrated into the plant chromosomes and subsequently lost. Some T-DNA may be converted to a double-stranded form prior to integration (28). Expression may be possible from such extrachromosomal elements. It is also possible that extrachromosomal T-DNA is integrated into other plant replicons such as chloroplast DNA and lost from the plant through loss of that replicon. Loss of T-DNA after integration into the plant genome may be a common occurrence. In our study, loss of GFP activity in previously stably expressing cell lines was attributable to both gene silencing and loss of T-DNA. Loss of T-DNA from stably expressing cell lines presumably can result from removal of chromosomally integrated T-DNA. Risseeuw et al. (34) proposed that T-DNA loci are inherently unstable in cultured cells. They found T-DNA was deleted in up to 20% of calli grown from transgenic leaf protoplasts. Also, Marton et al. (20) observed high rates of genetic lesions associated with T-DNA transfer but independent of stable T-DNA loci. They attributed these genetic lesions to T-DNA integration and subsequent removal (20).

Transiently expressing protoplasts that first divided soon after co-cultivation were likely to maintain silenced transgenic loci. Transient expressers that divided relatively late after co-cultivation were less likely to retain transgenic loci. This result has never been previously reported and is worth exploring further. The simple cell tracking system developed here would provide an ideal experimental system for further examination of this phenomenon. Physiological factors (eg. cell division, DNA replication, metabolic activity) that might alter T-DNA integration or stability could be manipulated at the time of gene transfer and specific cell lines isolated using the system described here.

Why might the time of first division be associated with T-DNA maintainence? It is likely that the time of protoplast first division was related to the cell cycle stage at the time of T-DNA transfer. It has previously been reported that stable transformation and transient expression are cell-cycle dependent (23, 42). The frequency of stable T-DNA integration within an individual cell might depend on cell cycle phase at the time of T-DNA transfer. Transgene stabilization appears to be associated with both replication of genomic DNA (S-phase) and cell division (M-phase). It is not clear on which aspects of S-phase and M-phase T-DNA expression and stabilisation would be dependent (eg. DNA replication, high metabolic activity, breakdown of the nuclear membrane). In the experiment reported here, there was no apparent difference in the time of first division between protoplasts that expressed GFP and protoplasts that did not express GFP. Therefore, if time of first division reflects cell cycle stage at the time of co-cultivation, the probability of a cell expressing *gfp* shortly after co-cultivation was independent of cell cycle in our experiments. However, cell cycle stage may have influenced the number of T-DNA copies transferred to each cell or the efficiency with which they were maintained. The time of first division might be more indicative of cell activity than cell stage at the time of T-DNA transfer. Metabolically active cells might integrate T-DNA more readily than less active cells. However, if early dividers were initially above average proliferators, they eventually lost this characteristic as there was no apparent relationship between time of first division and size of proto-calli four weeks after co-cultivation.

In all three experiments we observed that transient expressers produced smaller calli than other cell types. Transiently expressing protoplasts produced significantly smaller 4 week old calli than non-expressing protoplasts (Table 4. 3. 2). The difference in calli size between transient and stable expressers was not significant at the 5% level. However, the difference in size was very high ("significant" at the 7% level). The consistent difference between GFP- and GFP+ cell lines and the significant difference between GFP- and untransformed cells leads to the conclusion that the GFP- cell lines grew more slowly than other cells. It is uncertain why the growth rate of transient expressers was aberrant. As mentioned above, it is possible that cells that were less active were more likely to fail to stabilize transferred T-DNA. If this were so, there would have been a correlation between size of calli at four weeks and the number of transgenic loci. We did not observe such a relationship. However, the disproportionate loss from the study of the slowest growing

calli prior to molecular analysis may have masked any relationship between calli size and integration frequency. It is also possible that genetic lesions or epigenetic phenomena associated with transient expression inhibited the growth rate of transient expressers.

A simple system was developed that made it possible to isolate, culture and monitor the expression status of individual cells following co-cultivation with a *gfp* T-DNA construct. Further research using this approach should allow a more complete understanding of early processes involved in T-DNA expression and stabilization. A better understanding of these processes could provide the basis for new strategies to improve the efficiency of plant transformation. Transient expression has been defined as extrachromosomal T-DNA expression as opposed to stable expression in which T-DNA is integrated stably into the host genome (42). However, transient expression examined here was primarily associated with cell death. Both transient and stable expressers lost GFP activity through gene silencing and total loss of T-DNA. While expression from extrachromosomal T-DNA may happen, I suggest that there is no reason to assume that the frequency with which cells lose expression shortly after co-cultivation reflects the frequency of cells failing to integrate any expressed T-DNA.

4. 5. References

1. Cluster PD, O'Dell M, Metzlaff M, Flavell RB: Details of T-DNA structural organisation from a transgenic *Petunia* population exhibiting co-suppression. Plant Molecular Biology 32: 1197-1203 (1996).

2. Conner JA, Tanikanjana T, Stein JC, Kandasamy MK, Nasrallah JB, Nasrallah ME: Transgene-induced silencing of S-locus genes and related genes in *Brassica*. The Plant Journal 11: 809-823 (1997).

3. De Buck S, Jacobs A, Van Montagu M, Depicker A: *Agrobacterium tumefaciens* transformation and cotransformation frequencies of *Arabidopsis thaliana* root explants and tobacco protoplasts. Molecular Plant-Microbe Interactions 11(6): 449-457 (1998).

4. Delores SC, Gardner RC: Analysis of the T-DNA structure in a large number of transgenic petunias generated by *Agrobacterium*-mediated transformation. Plant Molecular Biology 11: 365-377 (1988).

5. Eady CC, Lister CE, Suo Y, Schaper D: Transient expression of *uidA* constructs in *in vitro* onion (*Allium cepa* L.) cultures following particle bombardment and *Agrobacterium*-mediated DNA delivery. Plant Cell Reports 15: 958-962 (1996).

6. Flavell RB: Inactivation of gene expression in plants as a consequence of specific sequence duplication. Proceedings of the National Academy of Science USA 91: 3490-3496 (1994).

7. Gallo-Meagher M, Irvine JE: Effects of tissue type and promoter strength on transient GUS expression in sugarcane following particle bombardment. Plant Cell Reports 12: 666-670 (1993).

8. Gleave AP, Mitra DS, Mudge SR, Morris BAM: Selectable marker-free transgenic plants without sexual crossing: transient expression of *cre* recombinase and use of a conditional lethal dominant gene. Plant Molecular Biology 40:223-235 (1999).

9. Haseloff J, Siemering KR, Prasher DC, Hodge S: Removal of a cryptic intron and subcellular localization of green fluorescent protein are required to mark transgenic *Arabidopsis* plants brightly. Proceedings of the National Academy of Science USA 94: 2122-2127 (1997).

10. Hooykaas PJJ, Schilperoot RA: *Agrobacterium* and plant genetic engineering. Plant Molecular Biology 19: 15-38 (1992).

11. Hunold R, Bronner R, Hahne G: Early events in microprojectile bombardment: cell viability and particle location. The Plant Journal 5: 593-604 (1994).

12. Iglesias VA, Moscone EA, Papp I, Neuhuber F, Michalowski S, Phelan T, Spiker S, Matzke M, Matzke A: Molecular and cytogenetic analyses of stably and unstably expressed transgene loci in tobacco. The Plant Cell 9: 1251-1264 (1997).

13. Jain RK, Jain S, Wang B, Wu R: Optimisation of biolistic method for transient gene expression and production of agronomically useful transgenic basmati rice plants. Plant Cell Reports 15: 963-968 (1996).

14. Janssen B-J, Gardner RC: Localized transient expression of GUS in leaf discs following cocultivation with *Agrobacterium*. Plant Molecular Biology 14: 61-72 (1989).

15. Kapila J, De Rycke R, Van Montagu M, Angenon G: An *Agrobacterium*-mediated gene expression system for intact leaves. Plant Science 122: 101-108 (1997).

16. Liu CN, Li XQ, Gelvin SB: Multiple copies of *vir*G enhance the transient transformation of celery, carrot and rice tissues by *Agrobacterium tumefaciens*. Plant Molecular Biology 20: 1071-1087 (1992).

17. Li XQ, Liu CN, Ritchie SW, Peng J, Gelvin SB, Hodges TK: Factors influencing *Agrobacterium*-mediated transient expression of *gusA* in rice. Plant Molecular Biology 20: 1037-1048 (1992).

18 Lorz H, Larkin PJ, Thomson J, Scowcroft WR: Improved protoplast culture and agarose media. Plant Cell Tissue and Organ Culture 2: 217-226 (1983).

19. Mandal A, Lang V, Orczyk W, Palva ET: Improved efficiency for T-DNA-mediated transformation and plasmid rescue in *Arabidopsis thaliana*. Theoretical and Applied Genetics 86: 621-628 (1993).

20. Marton L, Hrouda M, Pecsvaradi A, Czako M: T-DNA-insert-independent mutations induced in transformed plant cells during *Agrobacterium* co-cultivation. Transgenic Research 3: 317-325 (1994).

21. Matzke MA, Matzke AJ: How and why do plants inactivate homologous (trans)genes? Plant Physiology. 107: 679-685 (1995).

22. Maximova SN, Dandekar AM, Guiltinan MJ: Investigation of *Agrobacterium*-mediated transformation of apple using green fluorescent protein: high transient expression and low stable transformation suggest that factors other than T-DNA transfer are rate-limiting. Plant Molecular Biology 37: 549-559 (1998).

23. Meyer P, Walgenbach E, Bussmann K, Hombrecher G, Saedler H: Synchronized tobacco protoplasts are efficiently transformed by DNA. Molecular and General Genetics 201: 513-518 (1985).

24. Mozo T, Hooykaas PJJ: Factors affecting the rate of T-DNA transfer from *Agrobacterium tumefaciens* to *Nicotiana glauca* plant cells. Plant Molecular Biology 19: 1019-1030 (1992).

25. Nam J, Matthysse AG, Gelvin SB: Differences in susceptibility of *Arabidopsis* ecotypes to crown gall disease may result from a deficiency in T-DNA integration. The Plant Cell 9: 317-333 (1997).

26. Nam J, Mysore KS, Gelvin SB: *Agrobacterium tumefaciens* transformation of the radiation hypersensitive *Arabidopsis thaliana* mutants *uvh1* and *rad5*. Molecular Plant-Microbe Interactions 11(11): 1136-1141 (1998).

27. Narasimhulu SB, Deng X, Sarria R, Gelvin SB: Early transcription of *Agrobacterium* T-DNA genes in tobacco and maize. The Plant Cell 8: 873-886 (1996).

28. Offringa R, de Groot MJA, Haagsman HJ, Does MP, van den Elzen PJM, Hooykaas PJJ: Extrachromosomal homologous recombination and gene targeting in plant cells after *Agrobacterium*- mediated transformation. The EMBO Journal 9: 3077-3084 (1990).

29. Palauqui JC, Balzergue S: Activation of systemic silencing by localised introduction of DNA. Current Biology 9: 59-66 (1999).

30. Palauqui JC, Vaucheret H: Transgenes are dispensable for the RNA degradation step of cosuppression. Proceedings of the National Academy of Science USA 95: 9675-9680 (1998).

31. Pang S-Z, Jan F-J, Carney K, Stout J, Tricoli DM, Quemada HD, Gonsalves D: Post-transcriptional transgene silencing and consequent tospovirus resistance in transgenic lettuce are affected by transgene dosage and plant development. The Plant Journal 9: 899-909 (1996).

32. Park YD, Papp I, Moscone EA, Iglesias VA, Vaucheret H, Matzke AJM, Matzke MA: Gene silencing mediated by promoter homology occurs at the level of transcription and results in meiotically heritable alterations in methylation and gene activity. The Plant Journal 9: 183-194 (1996).

33. Payne RW: Procedure probit analysis. In: Payne RW, Arnold GM, Morgan GW (eds), Genstat 5 Release 4.1 Procedure Library Manual PL10. Numerical Algorithms Group Ltd, Oxford (1997).

34. Risseeuw E, Franke-van Dijk M, Hooykaas PJJ: Gene targeting and instability of *Agrobacterium* T-DNA loci in the plant genome. The Plant Journal 11: 717-728 (1997).

35. Ruiz MT, Voinnet O, Baulcombe DC: Initiation and maintenance of virus-induced gene silencing. The Plant Cell 10: 937-946 (1998).

36. Sambrook J, Fritsch EF, Maniatis T: Molecular Cloning. A Laboratory Manual. Ed. 2. Cold Spring Harbour Laboratory Press, (1989).

37. Schmulling T, Rohrig H: Gene silencing in transgenic tobacco hybrids: frequency of the event and visualisation of somatic inactivation pattern. Molecular and General Genetics 249: 375-390 (1995).

38. Sheng J, Citovsky V: *Agrobacterium*-plant cell DNA transport: have virulence proteins, will travel. The Plant Cell 8: 1699-1710 (1996).

39. Sonti RV, Chiurazzi M, Wong D, Davies CS, Harlow GR, Mount DW, Signer ER: *Arabidopsis* mutants deficient in T-DNA integration. Proceedings of the National Academy of Science USA 92: 11786-11790 (1995).

40. Stam M, Mol JNM, Kooter JM: The silence of genes in transgenic plants. Annals of Botany 79: 3-12 (1997).

41. Vergunst AC, Hooykaas PJJ: Cre/lox-mediated site-specific integration of *Agrobacterium* T-DNA in *Arabidopsis thaliana* by transient expression of *cre*. Plant Molecular Biology 38: 393-406 (1998).

42. Villemont E, Dubios F, Sangwan RS, Vasseur G, Bourgeois Y, Sandwan-Norreel BS: Role of the host cell cycle in the *Agrobacterium*-mediated genetic transformation of *Petunia*: evidence of an S-phase control mechanism for T-DNA transfer. Planta 201: 160-172 (1997).

43. Voinnet O, Vain P, Angell S, Baulcombe DC: Systemic spread of sequence-specific transgene RNA degradation in plants is initiated by localized introduction of ectopic promoterless DNA. Cell 95: 177-187 (1998).

44. Werr W, Lorz A: Transient gene expression in a gramineae cell line. A rapid procedure for studying plant promoters. Molecular and General Genetics 202: 471-475 (1986).

45. Ye F, Signer ER: RIGS (repeat-induced gene silencing) in *Arabidopsis* is transcriptional and alters chromatin configuration. Proceedings of the National Academy of Science USA 93: 10881-10886 (1996).

46. Yoshioka Y, Takahashi Y, Matsuoka K, Nakamura K, Koizumi J, Kojima M, Machida Y: Transient gene expression in plant cells mediated by *Agrobacterium* tumefaciens: application for the analysis of virulence loci. Plant Cell Physiology 37(6): 782-789 (1996).

Chapter 5

General Discussion.

5. 1. General discussion

At the inception of this research, three models were proposed to account for the transient nature of gene expression following T-DNA transfer to plant cells:

Model 1 Expression from the transferred DNA ceases when the T-DNA is lost.

Model 2 Loss of expression is caused by plant cell death.

Model 3 Expression is turned off epigenetically.

Simple predictions can be made from each of these models. If expression were lost through death of the transformed cells then, obviously, transient expressers would die shortly after gene transfer. If expression were lost through loss of T-DNA, then tissue derived from transient expressers would not contain T-DNA sequences. Neither cell death nor gene silencing was predicted to result in the elimination of T-DNA sequences. However, the possibility that expression was lost by gene silencing prior to loss of T-DNA sequences could not be tested. If expression was lost through gene silencing of stably integrated T-DNA, then tissue derived from transient expressers would contain copies of the T-DNA. The results presented in this thesis demonstrate that such simple models cannot individually explain transient expression. Of all cell lines that were transiently expressing the *gfp* gene 4 days after T-DNA transfer (Chapter 4), 64% died. Of the 17 surviving transient expressers examined, 10 (59%) retained some T-DNA sequences and 7 (41%) lost all T-DNA sequences. Gene silencing was demonstrated in 9 of the 10 cell lines that retained T-DNA sequences. The results demonstrate that in most surviving cells expression was lost by gene silencing. Where transient expression was associated with T-DNA loss, it is not known whether the expressed T-DNA was integrated into the plant genome or not.

The current model of transient expression after T-DNA transfer was derived by analogy with the model of transient expression after double-stranded plasmid transfer (6). Plasmid

DNA transfered to plant cells is, it is thought, expressed extrachromosomally. Some plasmid DNA integrates into plant genomic DNA and some does not. DNA that is not integrated is eventually degraded preventing further expression. The observation that transient expression also occurred after T-DNA transfer led to assumptions about expression of T-DNA. T-DNA is primarily transferred as single-stranded DNA, but expression is from double-stranded DNA. The accepted model of transient T-DNA expression is that single-stranded, extrachromosomal T-DNA is made double-stranded in the plant nucleus, expressed and then degraded without integration into the plant genome (6). However, there is no direct evidence that extrachromosomal, single-stranded T-DNA is made double-stranded. The data availible are equally compatible with transient T-DNA expression being from T-DNA that is integrated into the plant genome. It should be possible to decide whether transient expression was from unintegrated, chromosomal, or plastid T-DNA. T-DNA carrying a strong plant promoter directing transcription outward from near the left or right border could be transferred to plant cells. Analysis of mRNA a few days after co-cultivation could distinguish (by transcript length and sequence) between transcription from chromosomal T-DNA, unintegrated T-DNA or T-DNA integrated into plastid DNA.

There are two transient expression phenomena. One is the flux in enzyme activity after T-DNA transfer and the other is the change in the number of cells expressing the T-DNA. It is demonstrated here that the change in the number of cells expressing a T-DNA gene within 4 weeks of T-DNA transfer, is the result of three processes and is not predominantly due to T-DNA loss. However, it is still quite possible that the flux in T-DNA transcription/enzyme synthesis/enzyme activity that is observed as an average measurement from a sample of cells is substantially due to expression and loss of extrachromosomal T-DNA.

A principle role for gene silencing in the change in the frequency of recovered cells expressing the T-DNA is strongly suggested by the research presented here. The observed frequency of gene silencing (nearly 60% of surviving transient expressers) is certainly an underestimation of the true frequency as cell death and total T-DNA loss potentially obscure prior silencing events. The reverse is not true but some T-DNA was probably lost from transient expressers that lost *m-gfp5-ER* expression through gene silencing. That T-

DNA loss, in some transient expressers, occurred after T-DNA integration is suggested by the observation that T-DNA loss was not restricted to transient expressers. 26 out of 33 stable expressers cultured without selection regularly produced sectors lacking GFP activity. Southern analysis of 7 of these cell lines found evidence of T-DNA loss in 3. This result suggests that integrated T-DNAs are regularly lost during culture. An alternative explanation is that T-DNA integration occurred after division of the recipient protoplasts and the resulting cell lines were mosaics of transformed and untransformed cells. However, cell lines were maintained for many months with weekly subculture. During subculture, cells from GFP positive sectors only were selected. By this process untransformed cells would be rapidly lost from a mixture of transformed and untransformed cells. Yet sectors with no GFP activity regularly occurred from some cell lines over the months of culture. It is clear from these results that integrated T-DNA is unstable during cell culture as has previously been reported (8, 11). Where T-DNA was not detected in transient expressers, T-DNA loss could have occured at any time prior to DNA isolation and cannot be specifically associated with transient expression. Similarly, while death of transient expressers occurred within 2 weeks of the initiation of co-cultivation, those cells possibly had already lost transcription from the *m-gfp5-ER* gene by silencing or T-DNA loss.

I put forward a model of transient T-DNA expression to explain the rapid decrease in the frequency of plant cells expressing T-DNA within a few weeks of co-cultivation. I suggest that after T-DNA transfer to the plant nucleus there is expression from T-DNA (chromosomal and possibly extrachromosomal). Gene silencing is initiated in some plant cells and is passed to neighbouring cells by symplastic transport of a propagable silencing signal (13). Silencing might also be initiated in and passed on from cells that receive but do not express T-DNA. Subsequently, some cells die and there is an ongoing process of initiation of gene silencing and T-DNA loss in viable cells. Gene silencing, cell death and T-DNA loss all contribute to the rapid flux in the frequency of cells expressing transgenes after T-DNA transfer.

The concept that gene silencing can be a major cause of the transient nature of expression after T-DNA transfer is proven by the results presented here (Chapter 4). An unproven, novel, aspect of this model is the suggestion that a transmitted silencing signal might be partly responsible for the extent of loss of expression. I put forward this idea as we know

that gene silencing can be transmitted from cell to cell (13) and it is one possible explanation for the observation (Chapter 3, section 3. 1) that sometimes, after DNA transfer, transgene expression can only be observed in (symplastically isolated) stomatal guard cells. This part of the model could easily be tested, as the magnitude of loss of expression should depend on the extent to which transformed cells are symplastically connected.

The observation that gene silencing and cell death contribute to loss of expression has implications for research that uses transient expression. Transient expression is used as a measure of T-DNA transfer and the difference in the frequency of transient to stable expression is used as a measure of integration efficiency. Assumptions about transient expression have been important in research into the processes of T-DNA transfer, expression and integration. Also, assumptions about the mechanism of transient expression are of practical importance to researchers trying to optimise stable expression. Also, increasingly, researchers are using transient expression itself for processes such as gene tagging and molecular farming (manufacture and purification of specific chemicals that result from expression of foreign genes in transformed tissue).

The results presented here suggest that it may not be appropriate for researchers to use transient expression as a comparative estimate of T-DNA transfer. There is no experimental evidence demonstrating that two tissues with the same frequency/quantity of transient expression have received approximately the same amount of T-DNA. The frequency/quantity of transient expression probably reflects both the quantity of transfer and the number of plant cells competent to receive and express the T-DNA. It may be true that transient expression is limited by the quantity of T-DNA transfer. Equally, cell competence for expression might be limiting. Pre-culturing *H. aurantiacum* leaf discs on medium supplemented with phytohormones prior to co-cultivation with *A. tumefaciens* significantly increased the frequency of transient expression (Chapter 3). As the same effect was observed with DNA transfer by particle bombardment, it seems unlikely that the effect of pre-culture was to increase the quantity of T-DNA transfer. This result suggests that T-DNA might be transferred to more cells than it is expressed in. T-DNA transfer may not limit transient expression.

Several research groups have used the ratio of transient expression to stable expression as a measure of the frequency of T-DNA integration (eg. 12). This research suggests that gene silencing and cell death are at least as important as T-DNA loss in stopping expression. In fact, the assumption that the difference between transient and stable expression is T-DNA integration has no direct experimental support. A more direct way to compare the rate of T-DNA integration between two systems would be to extract DNA from plant cells periodically after co-cultivation, separate chromosomally integrated T-DNA from unintegrated T-DNA and quantitate T-DNA in each fraction by hybridisation to a labelled probe or by PCR. This method would still confuse differences in stability of integration with differences in the frequency of integration.

Researchers trying to improve the frequency of stable plant transformation usually adjust transformation parameters to optimise transient expression. This strategy is based on the assumption that if more DNA can be transferred to more cells then there is a higher probability that DNA will be integrated into the genome of regenerating cells. This is probably a useful strategy in systems where DNA transfer and integration limit stable transformation. However, higher frequencies of transient expression may not lead to more stable transformation in some systems for two reasons. Firstly, additional copies of the transferred DNA in each cell may increase the probability of gene silencing (1, 2, 10, 14). Secondly, the more cells that are exposed to the transferred DNA the more opportunity for gene silencing to be initiated in the tissue. Once initiated, gene silencing can be transmitted through the symplasm to neighbouring cells (13). Where high levels of transient expression do not lead to a high frequency of stable expression it might be sensible to try the reverse strategy and limit DNA transfer.

Interestingly, providing a dedicated integration system improved the frequency of stable transformation (4, 5, 7). Lebel et al (7) reported a 4-fold increase in the frequency of stable transformation when *Ds* transposition was used to integrate plasmid DNA into the plant chromosome. Integration of foreign DNA into the plant genome by *Ds* transposition might have had two effects. Firstly, transposition might have increased the frequency of DNA integration. Secondly *Ds* transposition might have increased the stability of integrated DNA. It has been suggested that plants integrate DNA by illegitimate recombination at genetically unstable sites (11). Certainly, the results reported here and elsewhere (8, 11)

suggest that DNA that is integrated into the plant genome by the normal host processes is highly unstable during tissue culture. It is possible that *Ds* transposition increased the frequency of stable transformation by not integrating at genetically unstable sites. It has been suggested that DNA integration sites become unstable during tissue culture (11). More research into the stability of integrated DNA is urgently required as T-DNA stability has important implications for emerging plant transformation technologies. Currently, transformation is primarily used to introduce single, dominant genes linked to a selectable marker gene into the plant genome. The stability of the transferred DNA during tissue culture is not a critical problem because cells that lose the transferred DNA are destroyed through the selection process. It is highly unlikely that this limited application is the future of plant transformation. Increasingly, germplasm enhancement will require the addition of genes to already transformed tissue. Transferred genes will not remain linked to selectable genes and the stability of previously transferred genes during transformation with new genes will be important. Alternative transformation strategies (such as transposition, site-specific integration and homologous recombination) will need to be developed to find techniques that minimise genetic mutation during transformation and ensure the stability of the integrated DNA. It will also be important to develop transformation systems that do not involve tissue culture to avoid excessive mutation during transformation.

Models of transient expression also have implications for researchers seeking to use transient expression to effect a change in the target cell or for molecular farming. Here transient expression of the *Ac* transposase gene was used to mobilize a *Ds* element for gene tagging in *H. aurantiacum* (Chapter 3). This strategy depended on the assumption that transiently expressed T-DNA would be lost from the cells. If expression was more often stopped by gene silencing than by T-DNA loss, then the results of the *Ds* transposition experiments (most spectinomycin resistant plants lost T-DNA marker gene expression but retained T-DNA sequences) are not surprising. As silencing of the marker gene does not ensure silencing of the *Ac* transposase gene, the usefulness of this gene tagging strategy, in its current state of development, is questionable. A similar strategy was employed to remove chromosomal DNA flanked by *lox* sequences in tobacco (3). In that strategy, the T-DNA carried the *cre* recombinase gene which catalyses recombination at *lox* sequences. The T-DNA also carried the conditional lethal, dominant marker gene *cod*A. After transfer of the T-DNA, 6 regenerated plants had evidence of recombination mediated by *cre*

recombinase and lacked *cod*A expression. Similarly to my gene tagging experiments, 4 out of the 6 plants retained the *cre* T-DNA. It may be generally true that after T-DNA transfer, selection for cells that do not express a marker gene on the T-DNA may not provide an effective mechanism to eliminate cells carrying the T-DNA. Similarly, researchers who seek to optimise transient expression to maximise enzyme production should not ignore gene silencing. Increasing the quantity of transient expression might be achieved by applying the cytosine-methylation inhibiter 5-azacytidine to *A. tumefaciens* prior to T-DNA transfer or the plant tissue after transfer, thus reducing gene silencing immediately after T-DNA transfer (9).

5. 2. References

1. Cluster PD, O'Dell M, Metzlaff M, Flavell RB: Details of T-DNA structural organisation from a transgenic petunia population exhibiting co-suppression. Plant Molecular Biology 32: 1197-1203 (1996).

2. Delores SC, Gardner RC: Analysis of the T-DNA structure in a large number of transgenic petunias generated by *Agrobacterium*-mediated transformation. Plant Molecular Biology 11: 365-377 (1988).

3. Gleave AP, Mitra DS, Mudge SR, Morris BAM: Selectable marker-free transgenic plants without sexual crossing: transient expression of *cre* recombinase and use of a conditional lethal dominant gene. Plant Molecular Biology 40: 223-235 (1999).

4. Houba-Herin N, Domin M, Leprince A-S: Some features about transposition of the maize element *Dissociation* in *Nicotiana plumbaginifolia*. Genetica 93: 41-48 (1994).

5. Houba-Herin N, Domin M, Pedron J: Transposition of a *Ds* element from a plasmid into the plant genome in *Nicotiana plumbaginifolia* protoplast-derived cells. The Plant Journal 6: 55-66 (1994).

6. Janssen B-J, Gardner RC: Localized transient expression of GUS in leaf discs following cocultivation with *Agrobacterium*. Plant Molecular Biology 14: 61-72 (1989).

7. Lebel EG, Masson J, Bogucki A, Paszkowski J: Transposable elements as plant transformation vectors for long stretches of foreign DNA. Theoretical and Applied Genetics 91: 899-906 (1995).

8. Marton L, Hrouda M, Pecsvaradi A, Czako M: T-DNA-insert-independent mutations induced in transformed plant cells during *Agrobacterium* co-cultivation. Transgenic Research 3: 317-325 (1994).

9. Palmgren G, Mattson O, Okkels F: Treatment of *Agrobacterium* or leaf disks with 5-azacytidine increases transgene expression in tobacco. Plant Molecular Biology 21: 429-435 (1993).

10. Pang S-Z, Jan F-J, Carney K, Stout J, Tricoli DM, Quemada HD, Gonsalves D: Post-transcriptional transgene silencing and consequent tospovirus resistance in transgenic lettuce are affected by transgene dosage and plant development. The Plant Journal 9: 899-909 (1996).

11. Risseeuw E, Franke-van Dijk M, Hooykaas PJJ: Gene targeting and instability of *Agrobacterium* T-DNA loci in the plant genome. The Plant Journal 11: 717-728 (1997).

12. Rossi L, Hohn B, Tinland B: Integration of complete transferred DNA units is dependent on the activity of *virulence* E2 protein of *Agrobacterium tumefaciens*. Proceedings of the National Academy of Science USA 93: 126-130 (1996).

13. Voinnet O, Vain P, Angell S, Baulcombe DC: Systemic spread of sequence-specific transgene RNA degradation in plants is initiated by localised introduction of ectopic promoterless DNA. Cell 95: 177-187 (1998).

14. Ye F, Signer ER: RIGS (repeat-induced gene silencing) in *Arabidopsis* is transcriptional and alters chromatin configuration. Proceedings of the National Academy of Science USA 93: 10881-10886 (1996).